ON THE SULPHUR PRESENT IN THE SEEDS OF CERTAIN LEGUMES, WITH SPECIAL REFERENCE TO THE CYSTINE PLUS CYSTEINE AND METHIONINE CONTENTS OF THE PROTEINS

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Of the legumes, those which produce large edible seeds have long been cultivated to provide a grain crop which is usually fairly rich in protein. It was inevitable, therefore, that the proteins contained in these seeds should have engaged the attention of investigators even in the early days of protein chemistry. More than sixty years ago Ritthausen had examined the properties of numerous protein preparations from the seeds of legumes and had shown that the sulphur contents were frequently low; and Osborne, who vastly extended Ritthausen's work with legume seeds, was able to demonstrate that many of their proteins were of low sulphur content.

Several years ago we (Lugg and Weller, 1941) found that the methionine content of the somewhat arbitrarily defined "protein" fraction of the embryos of ripe seeds of *Lupinus angustifolius*, was very low. Baernstein (1932) had earlier shown the methionine content of arachin, the main globulin of the seeds of *Arachis hypogea*, to be very low; and White and Beach (1937-8) had shown that the poor nutritive value of arachin as a rat food was to be associated with its low methionine content. The question thus arose whether the methionine contents, not only of the proteins, but of the rest of the edible material, were too low to justify the legume seeds in general being regarded as satisfactory sources of protein and amino-acids in the nutrition of animals.

Since then, Brown (1942) has quoted a slightly greater methionine content for arachin than that found by Baernstein (1932). Klose and Almquist (1941) have shown that considerable addition of methionine (or of homocystine plus choline) was needed for optimal growth of chicks maintained on a diet in which the great bulk of the protein was represented by arachin. Hayward and Hafner (1941) have shown that for the satisfactory nutrition of chicks and rats the protein of soya beans (*Glycine soja*) requires supplementing with methionine. Almquist, Mecchi, Kratzer and Grau (1942) too, have found that with chicks the main growth-limiting deficiency in raw and cooked soya beans, is methionine.

In the present study an attempt has been made to assess the nutritive values of the seeds of several legumes from the standpoint of their methionine (and, to a lesser extent, of their cystine and/or cysteine) contents. The work was confined to the New Zealand (or Mediterranean) Blue Lupin (*Lupinus angustifolius*), the Western Australian Blue Lupin *(L. pilosus* sub. sp. *Cosentini)*[1], the Yellow Lupin *(L. luteus)*, peas *(Pisum sativum*, var. *Greenfeast)*, and "haricot" beans *(Phaseolus vulgaris)*.

In Australia, Greenfeast peas are normally used in the unripened, undried condition in human diets and are cooked (boiled or steamed) for consumption. Haricot beans customarily (and Greenfeast peas, occasionally) come into use in a ripened air-dry condition. They are soaked in water for some time before use and are then cooked. The lupin seeds appear to be rarely (if ever) employed here in human diets, but in recent decades the Western Australian Blue Lupin, in particular, has found considerable exploitation as a stock food in certain parts of Western

[1] From Fiori's key. Also frequently designated, "*L. varius*".

Australia which do not otherwise readily furnish legume pasturage or pulse crops. The foliage contains a bitter principle and is not ordinarily eaten by the stock, but the ripe, dry seeds are avidly consumed.

These considerations, together with the fact that in human diets some other varieties of *Pisum sativum* seeds are usually ripened and dried before use and some varieties of *Phaseolus vulgaris* seeds are consumed together with edible pods in an unripened, undried condition, rendered it desirable to acquire some knowledge of the changes (if any) in the sulphur distributions occasioned by the ripening and drying of legume seeds. This aspect of the matter was examined with the New Zealand Blue Lupin and with Greenfeast peas.

EXPERIMENTAL.

Preparation of Materials for Analyses.

In the earlier work with *L. angustifolius* seeds (Lugg and Weller, 1941), the testas were removed, and a "whole" protein preparation was obtained from the ground-up embryos by extracting what were deemed to be the non-protein nitrogenous constituents and, incidentally, various non-nitrogenous substances. The testas were shown to contain very little nitrogen.

It was intended to follow the same procedure with the *L. pilosus* and *L. luteus* seeds, but when the method of removing the non-protein nitrogenous constituents employed by Lugg and Weller (1941) was applied to the *L. pilosus* embryos, a large part of the nitrogen appeared in the un-coagulated, water-soluble fraction at pH 4·5. Addition of alcohol to the extract brought down a copious precipitate, and it was evident that a considerable amount of material, which would reasonably be classed as "protein", had escaped into the extract. So recourse was had to a modification of the procedure described by Lugg (1939a), in which the first extraction is made, not with acidified water itself, but with acidified water to which has first been added 0·8 vol. of alcohol. This procedure has been found to give substantially the same results as the other with dried leaf material containing almost entirely denatured protein, and it is known that the native leaf proteins are far less soluble in the alcoholic aqueous extractant than they are in the aqueous extractant. When the procedure was applied to the *L. pilosus* embryos, only a small fraction of the total nitrogen was found to be non-coagulable. The procedure was modified in that instead of using an aqueous citric acid solution in a subsequent extraction of the coagulum, there was used an aqueous solution of Na citrate at pH 4·5 to which had first been added 0·8 vol. of alcohol. In obtaining "whole" protein preparations from the other embryos and seeds, this was the method used. A new "whole" protein preparation was obtained by the same method from the embryos of ripe, dry *L. angustifolius* seeds.

The unripe *P. sativum* and *L. angustifolius* seeds were removed from the pods, split through the hilum, and dried as rapidly as possible to about 10 p.c. moisture content, in a current of air at 80° C. The peas had been harvested in a distinctly "tender" condition, and the weight of the fresh seeds themselves was only 38 p.c. of the weight of the full pods. The unripe seeds of *L. angustifolius* had been harvested in what seemed to be a similar condition.

The testas were removed from all the lupin seeds, and subsequent analytical work was largely confined to the embryos and to "whole" protein preparations obtained therefrom. Testas were not removed from the peas or the haricot beans.

TABLE 1.

Particulars concerning the original seeds.

Seed with date of initial treatment*.	Per 100 seeds†. Fresh wt. gm.	Dry wt. gm.	P.c. N.	Dry wt. testas gm.	P.c. N testas.	Dry wt. embryos. gm.	P.c. N embryos
Lupinus angustifolius (unripe) 16/12/'41	29·0	(9·00)	(5·35)	2·96	1·09	6·04	7·44
Lupinus angustifolius (ripe) 21/1/'44	22·4	(20·6)	(5·53)	4·4	0·50	16·2	6·90
Lupinus pilosus (ripe) 26/11/'41	19·6	(17·8)	(5·41)	5·16	0·45	12·6	7·44
Lupinus luteus (ripe) 15/8/'41	10·8	(9·60)	(6·90)	2·54	0·78	7·06	9·10
Pisum sativum (unripe) 6/3/'42	50	11·4	5·08				
Pisum sativum (ripe) 2/6/'42	22·3	20·0	4·38				
Phaseolus vulgaris (ripe) 22/1/'44	25·4	22·6	3·21				

* i.e. powdering of seeds, or removal of testas and powdering of embryos. The dates are also the dates of harvest of the unripe seeds. The ripe seeds were harvested less than 1 year before the date of treatment.

† Values derived by calculation are shown in parentheses.

Further information which will serve to describe the original seed material is given in Table 1. In Table 2 are data concerning the preparation of "whole" protein samples from the embryos of the lupin seeds and from the entire material of the other seeds. The values given have been corrected for the very small errors to show perfect recovery of the extracted nitrogen in the extracting liquids.

TABLE 2.

Preparation of "whole" protein samples from seeds and embryos.

No.	Seed material.	Dry wt. taken gm.	P.c. N.	Total N mg.	Coagulable N mg.	Protein N mg.	Dry wt. protein gm.	P.c. N in protein prep.
1.	*Lupinus angustifolius* (unripe) embryos	5·46	7·44	406	324	320	3·24	9·86
2.	*Lupinus angustifolius* (ripe) embryos	6·15	6·90	424	389	387	4·30	9·01
3.	*Lupinus pilosus* (ripe) embryos	11·5	7·44	853	750	743	7·76	9·57
4.	*Lupinus luteus* (ripe) embryos	12·8	9·10	1,169	1,046	1,041	8·20	12·7
5.	*Pisum sativum* (unripe) seeds	9·01	5·08	458	284	272	4·97	5·46
6.	*Pisum sativum* (ripe) seeds	9·48	4·38	415	348	333	6·83	4·88
7.	*Phaseolus vulgaris* (ripe) seeds		3·21					

Methods of Estimation.

Nitrogen estimations were made by the Kjeldahl method. Sulphur distributions were determined by the "differential oxidation" method described by Lugg (1938), and by a minor modification of it in which the basic principles are unchanged. This modification is in respect of the "total sulphur" estimations only, the HNO_3 oxidations and the sulphate–S estimations being carried out as described before.

In the modified procedure for estimating total–S, 0·2 gm. of the material is treated with 20 mg. glucose[2] and 5 ml. 10 N NaOH, and the mass is evaporated to dryness at 105° C. with frequent pricking of the surface crust to facilitate loss of moisture. The mass is then cautiously heated with stirring until it fuses, and after being raised to dull redness is allowed to cool. The mass is then re-fused under 2 gm. of Na_2O_2 to dull redness and a further 0·1 gm. of Na_2O_2 is added during cooling. Subsequent treatment of the melt is as described before.

This "peroxide fusion" procedure is similar to one used by Osborne (1900). We have found it generally more reliable[3] than the one described before. Flaring (which far too frequently occurs in the previously described procedure, and ruins the estimation) seems not to occur at all.

RESULTS AND DISCUSSION.

The sulphur distributions are not shown as such in Table 3, but the cystine plus cysteine [cyst(e)ine] and methionine contents of the preparations calculated therefrom[4] and expressed as percentages of the nitrogen present occurring in these forms, are shown, together with the sulphate sulphur contents also expressed in relation to the nitrogen present.

The fundamental assumptions underlying these calculations are (1) that the sulphur present is contained exclusively in cyst(e)ine, methionine and sulphate, the amino-acids being present as such or as residues in the protein molecule, and (2) that the extent to which sulphate is produced by the action of the reagents upon the residues is the same as for the free amino-acids.

These assumptions have been discussed elsewhere (Lugg, 1938, 1939b). In our view there is still no indisputable evidence of the existence of other forms of sulphur in undegraded proteins. Accordingly, in the present state of our knowledge of proteins, we presume that the

[2] The glucose was added to ensure initial reducing conditions. It has not been proved necessary but its inclusion was suggested by the belief that partial oxidation of organically combined sulphur to highly unreactive compounds may occur rather easily.

[3] Satisfactory estimation of "total–S" by oxidation methods is far more difficult than is sometimes realized (see remarks by Masters, 1939). We found that combustion with oxygen in the calorimeter bomb generally did not effect oxidation of all the sulphur to sulphuric acid. This is at variance with Marston's (1937) experience, but he customarily used much larger quantities of material than we did.

[4] It becomes increasingly important, as the cyst(e)ine–S : methionine–S ratio differs from unity, to make careful allowance in the calculations for the few per cent. of cyst(e)ine–S not oxidized to H_2SO_4 and for the few per cent. of methionine–S oxidized to H_2SO_4, during the digestion with HNO_3.

cyst(e)ine and methionine contents of the "whole" protein preparations, shown in the Table, are not vitiated by the existence of forms of sulphur ignored in the assumptions. It is also presumed, from such evidence as does exist (Lugg, 1938), that the values are substantially free from systematic errors. But the first assumption is known to be inapplicable to the entire material of at least some seeds. Thus mustard oils and djenkolic acid, which occur in certain seeds, and thiamine, which occurs in small quantities in many seeds, are sulphur-containing compounds ignored in the assumptions. The values shown in the Table for the unextracted seeds and embryos have been printed in italics to indicate that the results may possibly be vitiated by the inclusion of unspecified compounds of sulphur.

The cyst(e)ine and methionine contents of one whole protein preparation were estimated also by the modified Baernstein procedure (HI digestion-titration) described by Lugg (1938), and are shown in a footnote to Table 3. The known deficiencies of this procedure with impure proteins may be held to account for the rather lower values obtained.

With the exception of a few of the sulphate values, the quantities shown are the means of closely agreeing duplicate and sometimes triplicate estimations, and the random errors of estimation are believed to be not greater than the following: 0·01–0·05 in the sulphate values, 0·02–0·04 in the cyst(e)ine values, and 0·03 in the methionine values (the larger errors being associated with the larger sulphate values).

TABLE 3.

Partial compositions of the preparations: Losses during extraction.

No.*	P.c. N.	P.c. Cyst. N.	P.c. Meth. N.	$\frac{SO_4\text{–}S}{N} \times 100.$	P.c. loss, or apparent loss, during extraction.		
					Cyst.	Meth.	SO_4–S.
1	7·44	*1·02*	*0·31*	3·18	14	— 10	95
1W	9·86	1·12	0·43	0·22			
2	6·90	*1·31*	*0·32*	1·62	1	26	79
2W	9·01	1·42	0·26	0·38			
3	7·44	*1·36*	*0·42*	0·13	0	0	67
3W	9·57	1·56	0·48	0·05			
4W	12·7	2·07†	0·37†	0·03			
5	5·08	*0·67*	*0·60*	2·05	27	21	99
5W	5·46	0·82	0·80	0·04			
6	4·38	*1·01*	*0·56*	0·39	40	5	84
6W	4·88	0·75	0·66	0·08			
7	3·21	*1·47*	*0·85*	0·19			

* The numbers are taken from Table 2. The suffix "W" signifies the "whole" protein preparation made from the particular seed material.

† Corresponding values obtained by the HI digestion-titration procedure were 1·60 and 0·29, respectively.

On the right of Table 3 are shown the apparent losses of cyst(e)ine and methionine and the losses of sulphate in the extraction process used to obtain the "whole" protein preparations. They have been calculated with the aid of the data shown in Table 2. Certain of the losses are necessarily qualified as "apparent" because of what has been stated about the possible vitiation of the cyst(e)ine and methionine values for the unextracted material.

The losses must also be viewed in the light of the magnitude of the random errors. Thus the random errors are insufficient to account for the cyst(e)ine loss in any instance, except in the preparation of 2W from 2, but the apparent "gain" (negative loss) of methionine in the preparation of 1W from 1, and the small loss in the preparation of 6W from 6, can be accounted for entirely on the basis of the magnitudes of the random errors.

It would seem that there could have been virtually no free cyst(e)ine in 2 and 3 and virtually no free methionine in 3. Likewise there could have been virtually no other sulphur-containing compounds present to vitiate the cyst(e)ine values for 2 and 3 and the methionine value for 3.

Whether, having allowed for the random errors, any portions of the remaining "apparent" losses of cyst(e)ine and methionine were real, in the sense that they could not be attributed to the presence of unspecified sulphur-containing compounds in the unextracted materials, it is impossible to say.

The composition differences between the "whole" proteins of the ripe and unripe *P. sativum* seeds are not very great. There is a fairly pronounced difference, however, between the methionine values for the "whole" proteins of the ripe and unripe *L. angustifolius* embryos.

Whereas about 22 p.c. of the nitrogen present in the ripe *L. angustifolius* embryos examined by us earlier (Lugg and Weller, 1941) appeared to be non-protein nitrogen, only some 9 p.c. could have been non-protein nitrogen in the new ripe embryos. It is possible that at least part of this difference is attributable to the difference in methods of extracting the non-protein nitrogen. However, the cyst(e)ine and methionine contents of the "whole" protein of the new embryos are very similar to those reported earlier [1·16 p.c. cyst(e)ine N, 0·24 p.c. methionine N].

Whether the "whole" proteins of the other seeds and embryos examined exhibit small or great variability in composition, is not known. Unfortunately, there is little of the earlier work on variability of "whole" seed protein composition, in which the applicability of the methods then used would not now be regarded with doubt. There is much evidence as to the non-variability of composition of the individual proteins in many seeds. And although Osborne (1900) was of opinion that proteins like vicilin, phaseolin and conglutin, are not of constant composition, it must be realized that he frequently applied these names to samples of protein obtained from seeds belonging to different species of legumes.

On the other hand there is evidence of variability in the proportions of the individual proteins in seeds at different stages of maturity and produced under different conditions. See, for example, Bishop (1928, 1930) and Fink and Kunisch (1937a, b, c) for work with barley, and Woodman and Engledow (1924) and Grewe and Bailey (1927) for work with wheat.

This study was concerned primarily with the nutritional values of the seeds and of the seed proteins, and from this viewpoint even the italicized cyst(e)ine and methionine values may be accepted as "maximal" (to within the random errors). In this view, the low methionine values are particularly striking. The cyst(e)ine values are mostly of average magnitude: several are rather small—one is quite large.

It will be observed that with the lupin embryos and "whole" protein preparations, the cyst(e)ine values are fairly large, whereas the methionine values are really small. Of special interest is the very low methionine value for the "whole" protein of the embryos of the ripe seeds of *L. angustifolius*. Corresponding values with *L. luteus* and *L. pilosus* were about 1·4 and 1·85 times as great, respectively, and with the former, moreover, the cyst(e)ine value was about 1·45 times as great as that of the *L. angustifolius* preparation. Ritthausen, who gave the name "conglutin" to the characteristic protein of lupin seeds, stated that preparations from *L. luteus* and *L. angustifolius* seeds were scarcely distinguishable, except that the sulphur contents were 1 p.c. and 0·5 p.c. respectively (see Ritthausen, 1868, 1882). Osborne and Campbell (1897) indicated that purified preparations (fractions least soluble in NaCl solution) from these sources, containing 0·52 p.c. and 0·32 p.c. of sulphur respectively, differed only in that the former preparation was more heavily contaminated than the latter with a sulphur-rich impurity. Later, Osborne (1900) inclined to the view that these "conglutins" were mixtures of varying proportions of proteins similar in many respects but containing distinctly different quantities of sulphur.

With the pea seeds and "whole" protein preparations, the methionine values are larger and the cyst(e)ine values smaller, than those found for the lupins. With the haricot beans the "maximal" cyst(e)ine content is of average magnitude and the "maximal" methionine content is the largest for the group (though only 0·85 p.c. of the N.).

The digestibilities of the testas of the pulse seeds are likely to be low for many animals, but ruminants would probably digest them better than most. In any case Table 1 shows that very little of the total nitrogen was contained in the testas. Special attention was paid only to the testas of the ripe *L. pilosus* seeds. They contained 2·5 p.c. of the total nitrogen in the seeds and 1 p.c. of the total sulphur. If this sulphur had been contributed entirely by cyst(e)ine, it could have increased the cyst(e)ine content of the seeds by only about 1·5 p.c., and if it had been contributed entirely by methionine the methionine content of the seeds could have been increased by only about 4 p.c.

In his study of the sulphur present in proteins, Osborne (1900) digested proteins with alkali containing some plumbite and in the presence of zinc, to de-

termine "loosely-bound" sulphur. In some instances the "firmly-bound" moiety probably provided an estimate of the methionine present, but it might have afforded a far better estimate if the "loosely-bound" moiety had been estimated by a method like that of Zahnd and Clarke (1933) and had thereby provided a less questionable estimate of the cyst(e)ine present. In "conglutin" obtained from *L. luteus* and *L. angustifolius* seeds, Osborne found only about 2/3 of the total sulphur to be "loosely-bound". In "legumin" obtained from the horse bean, the vetch and the lentil, and in "vicilin" from the pea, the horse bean and the lentil, he found about 1/2 of the total sulphur to be "loosely-bound". In phaseolin from the kidney bean, he found about 1/4 of the total sulphur to be "loosely-bound". The above interpretation of Osborne's results for vicilin finds its correspondence in our results with the *P. sativum* preparations, but the accord between his results for the conglutins and our results for the lupin preparations, is less close.

In so far as methionine, free or combined in protein, is essential in the diet of an animal (in addition to references given earlier, see especially Womack, Kemmerer and Rose, 1937, and Rose, Haines and Johnson, 1942) the legume seeds we have examined, being rather poor to very poor sources in terms of the nitrogen present, cannot be regarded as of high nutritive value. The methionine contents, expressed as percentages of the nitrogen present, were low in comparison with those of the whole proteins of muscle, milk and plant leaves. The cyst(e)ine contents, in terms of the nitrogen present, varied from low to fairly high. As cyst(e)ine may exercise a sparing action in favour of methionine (see, in particular, Womack and Rose, 1941, and Madden, Carter, Kattus, Miller and Whipple, 1943) the seeds of higher cyst(e)ine content may be rather less unsatisfactory nutritionally than their low methionine contents (viewed in isolation) would make them.

In view of the findings here presented and with regard to the observations of others who have worked with legume seeds, it is suggested that the seeds and "whole" seed proteins of any legume should be deemed of low methionine content unless experimental evidence exists to the contrary.

SUMMARY.

The seeds of the New Zealand (or Mediterranean) Blue Lupin *(Lupinus angustifolius)*, the Western Australian Blue Lupin *(L. pilosus*, sub sp. *Cosentini)*, the Yellow Lupin *(L. luteus)*, peas *(Pisum sativum*, var. *Greenfeast)* and "haricot" beans *(Phaseolus vulgaris)* have been examined with regard to the sulphur distributions and with special reference to the cyst(e)ine and methionine contents of the "whole" proteins.

In the two instances studied, the ripening of somewhat immature seeds was not accompanied by vast changes in the cyst(e)ine or methionine contents of the "whole" proteins.

Relatively small amounts of cyst(e)ine and methionine could have occurred in the seeds other than in combination as amino-acid residues in the protein present.

In terms of the nitrogen present, the methionine contents were rather low to very low, and the cyst(e)ine contents were rather low to fairly high. The implications in regard to the nutrition of animals have been discussed.

It is suggested that in the absence of actual experimental evidence to the contrary, the seeds and "whole" seed proteins of any legume should be deemed of low methionine content.

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ON THE STIMULATING EFFECT OF ELECTRIC CURRENTS OF EXTREMELY SHORT DURATION

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Ever since the duration of electric currents has been recognized as one of the cardinal factors determining the exciting effect of electric stimuli (Fick, 1863; Weiss, 1901), a considerable amount of work has been done on the influence and significance of this factor. The classical observations by Weiss, Lucas (1906, 1907a, b and c, 1908) and Lapicque (1926) have contributed most to our experimental knowledge in this field. As these observations have an important bearing on the present problem a brief summary of them will first be given.

Between various durations of direct currents and the corresponding liminal voltages, i.e. the voltages required for minimal excitatory effects, there is a characteristic relationship. Fig. 1 shows the results of an experiment on the sciatic nerve of a frog. The liminal voltages plotted against the durations (strength (voltage)-duration curve) fall roughly upon a hyperbola (Weiss, 1901). The products of the durations and the corresponding liminal voltages represent the quantities of electricity necessary for minimal excitation. The graphic representation of these liminal quantities shows in another and often more conspicuous way the law governing electric stimulation. In the above example the quantity-duration curve is roughly a straight line,[1] crossing the ordinate somewhere above the origin—a point of importance for this paper.

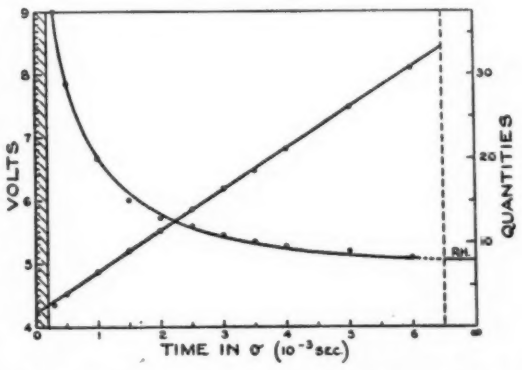

Fig. 1. Strength-duration curve and quantity-duration curve of the sciatic nerve of a frog. Shaded area marks the very short durations. Rh = rheobase.

The usual strength-duration curves for the nerve extend over a range of durations between about $0{\cdot}1\sigma$ and 8 to 10σ ($1\sigma = 1$ millisecond). Beyond the latter times the curve becomes parallel with the ordinate, i.e. there is a minimum effective voltage which cannot be further reduced, however long the current may last. This minimum voltage, identical with the absolute threshold of stimulation of the older physiology, has been termed by Lapicque the rheobase. The shortest duration at which the liminal voltage decreases to this lowest effective value has no practical importance.[2]. The liminal[3] duration of a current twice as strong as the rheobase is, however, important for practical and theoretical reasons. Both Lucas (1907a) and Lapicque (1909) have

[1] As will be discussed later, it is still uncertain whether the quantity-duration curve for the nerve is, within the range of durations of the above experiment, really a straight line or curved in some way. The quantity curve for the striped muscle seems to be S-shaped (Lippay, 1938).

[2] Gildemeister (1913) has called this duration of the galvanic current: "Hauptnutzzeit", i.e. main utilization time. Due to the flatness of the curve in this region this time cannot be determined with accuracy and has therefore been excluded from the ordinary experimental procedures.

[3] This means, of course, the shortest duration required to produce a minimal effect.

independently suggested the use of this duration, instead of the old threshold of stimulation, as a measure of excitability. Lucas called it excitation time, Lapicque proposed the term chronaxie, i.e. characteristic time. The latter expression is more widely used, although Lucas's concept of excitation time, being independent of any theoretical views, has general significance and was later, in some cases, also adopted by Lapicque[4] (1934) who wanted the term chronaxie restricted to those cases in which the strength duration curve conformed with what he considered to be the only true curve (canonical curve).[5]

Experimental evidence, due mainly to Lapicque and his school, shows that the chronaxie represents, in fact, a true measure of excitability, being essentially determined only by properties of the excitable structures themselves and not, as is the absolute threshold, by extraneous circumstances. Thus, the excitation time is generally found to be shorter in structures with quick reaction (e.g. the myelinated nerve fibre) and longer in structures with slow reactivity (e.g. plain muscle). Even at the present time full use has not been made of these far-reaching results, particularly in the field of electro-medicine, where the old method of testing muscle and nerve excitability by means of threshold values still prevails, no advance having been made towards a real discrimination and separate examination of nerve and muscle excitability on these modern lines.

As the chronaxie for the nerve is of the order of about $0{\cdot}3$–$0{\cdot}5\sigma$ and as strength-duration curves exhibit their characteristic shape above duration of $0{\cdot}2\sigma$, it is natural that duration below $0{\cdot}2\sigma$ have not received much attention, especially, as the production of such short currents meets with greater experimental difficulties. In fact, there are no systematic observations concerning extremely short galvanic currents, although some authors have, in one or the other case, included some in their experiments. Gildemeister and Weiss (1909) have probably gone farther in this direction than others (lowest duration tested $0{\cdot}013\sigma$), but their results do not seem to be very accurate and differ considerably from those presented in this paper. Occasional data referring to such very short durations in some papers are partly in agreement with those described in this paper.

Currents of very short durations thus not yet properly investigated can however be made a matter of speculation in extending to them the equations expressing the quantitative relation between time and intensity (voltage) of currents with liminal effect. Thus Weiss's empirical formula (Weiss, 1902) according to which the liminal quantities fall upon a straight line (Fig. 1) would postulate that there is a lower limit for the threshold quantities; while Lapicque's empirical formula (Lapicque, 1926), as well as Nernst's (1908) and Hill's (1910) formulae, both based on certain theoretical assumptions, state that, with decreasing durations, the threshold quantities decrease progressively. It should also be mentioned that Cremer, in a review of the laws of electric nerve stimulation (Cremer, 1929), expressed the view that available observations seemed to suggest the existence of a minimum in the effective quantity of liminal electric stimuli.

It will be seen from the foregoing discussion that the field of extremely short durations of stimulating currents (shaded field in Fig. 1) has been almost completely neglected. The experiments described in this paper have been undertaken to obtain, independently of any theoretical assumption, exact knowledge of the conditions of stimulation at those extremely short durations. The main questions to be examined in this connection are: Is there any regularity in the voltage-time relation of currents of extremely short duration (under $0{\cdot}2\sigma$)?; Is there a lower limit for the threshold quantities, comparable to the intensity threshold? Does any one of the formulæ for electric stimulation derived from observations at durations above $0{\cdot}2\sigma$ agree with the experimental findings at very short durations?

METHODS.

The experiments were made on the sciatic gastrocnemius preparation of the frog (Rana esc.). All preparations were first kept for about ½ hour in Ringer solution and then suspended in the moist chamber used in the experiments. To ensure stable conditions the preliminary tests of excitability were made after at least another ½ hour. The experiments proper were not undertaken unless several chronaxie estimations, made in intervals of 5–10 minutes, had proved the absence of any considerable variations of excitability.

For nerve stimulation thin silver wire electrodes, coated electrolytically with silver chloride, were used. The distance between the electrodes was 15 mm.

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[4] Lapicque's ET2R means: excitation time for voltages twice as strong as the rheobase. For practical purposes both expressions mean the same thing and can be used indiscriminately.

[5] Whether such curves do really occur in nerves is doubtful (Rushton, 1932).

The experiments are divided in two groups: one with galvanic currents, the other with condenser discharges as stimuli.

(a) *Stimulation with galvanic currents.* The stimulating current was obtained in the usual way by leading off from a main circuit. For this purpose a precision potentiometer, described elsewhere (Lippay, 1937) with special arrangements for chronaxie determination was used. The single resistances in this instrument are coils with induction and capacity free windings according to Wagner and Wertheimer (1913), ensuring changes of the current without delay—an indispensable condition for the production of rectangular waves. The total potentiometer resistance being 100 ohm., the preparation with its high residence can practically not affect the voltage distribution. The arrangement of three decade units (9×10, 9×1 and $10 \times 0{\cdot}1$ ohm., each single value accurate within $0{\cdot}02$ p.c.) made possible finer regulation of the stimulating current to any degree required in such experiments. An induction free resistance of more than 25,000 ohm. was in series with the nerve.

The voltage of the stimulating current was measured with a precision voltmeter (scale of 150 degrees, 4 ranges, internal resistance 1,000 ohm. per volt, indications correct within $0{\cdot}1$ p.c. of full scale value). For greater accuracy the ranges were so chosen that all readings could be taken in the higher parts of the scale. Readings being correct to within $0{\cdot}2$ of a degree, the errors in measuring the voltages could hardly have exceeded 1 p.c. of the actual value.

The duration of the stimulating current was controlled by a Helmholtz pendulum.[6]. In the usual way two break contacts were used, the first as a shunt to the preparation, the second as an interrupter of the preparation circuit. It is clear that the break of the first contact leads to instantaneous flow of the stimulating current through the nerve whilst the break of the second contact brings this current instantaneously to an end. As in these experiments correct functioning of the contacts was of primary importance, special attention was given to accurate calibration of the pendulum. This was done by the method of Radacovic (1900) (Edelmann, 1900), using induction-free resistances, mica condensers and a ballistic galvanometer, all instruments of highest precision. Calibrations were repeated from time to time. The main scale of the Helmholtz pendulum has 60 degrees and covers a time of about 18σ; as each unit of this scale corresponds to $0{\cdot}3\sigma$, and is further subdivided on a micrometer drum in 100 parts, it is possible to obtain intervals between the contact breaks as short as $0{\cdot}003\sigma$. Controls have shown that the shortest durations examined in the experiments were correct within reasonable limits.

(b) *Stimulation with condenser discharges.* The usual arrangement for this purpose (Lapicque, 1926; Laugier, 1929) was employed. The voltage for charging was obtained by means of an accurate dial potentiometer and measured with precision voltmeter. Accurate mica condensers divided in $10 \times 0{\cdot}1$, $10 \times 0{\cdot}01$ and $10 \times 0{\cdot}001$ micro farads were available, thus giving any required gradation of the capacity. The rheobase was determined by means of a high capacity (40 m. f.). The necessary connections for charging of the condensers and their discharging through the nerve were made with a Morse key.

Constant resistance in the discharge circuit was ensured in one group of the experiments with Lapicque's resistance combination (total resistance, irrespective of variations of the preparation resistance, nearly 10,000 ohms.) and in another group of experiments with Bourguignon's arrangement (total resistance around 11,000 ohms.), Bourguignon, 1923). In the first group there was an additional resistance of about 20,000 ohms. in series with Lapicque's arrangement, making a total resistance of about 30,000 ohms.

EXPERIMENTS AND RESULTS.

With the two methods described a greater number of experiments was carried out, the results of which are essentially the same. It is desirable to deal with the two kinds of experiments separately.

(a) *Experiments with Galvanic Currents.*

Twenty-five experiments are available. In each of them, after determination of the chronaxie, the threshold voltages for a series of certain durations, as shown in Table 1, were determined, proceeding gradually from the longer towards the shorter durations or vice versa. In most experiments the direction of the stimulating current in the nerve was "descending", i.e. from the central nervous system towards the periphery. As in some experiments with ascending currents the same results were obtained, the direction of the current is evidently unimportant.

The results obtained are best illustrated in the quantity-duration curves. Strength-duration curves, without complicated analysis, do not show satisfactorily the peculiarities under investigation and are, therefore, omitted.

[6] With this pendulum it is possible to obtain much shorter durations than with the Lucas pendulum which cannot give durations far below $0{\cdot}1\sigma$.

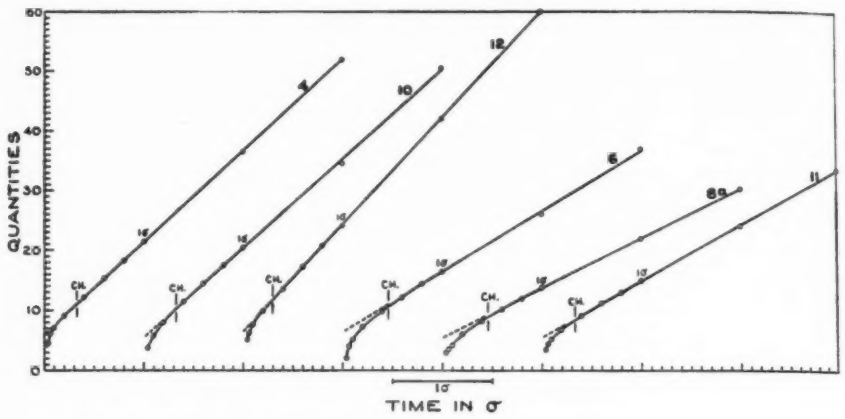

Fig. 2. Quantity duration curves of six experiments. Ch = chronaxie. Compare Table 1.

Fig. 2 shows six examples of typical quantity curves. The experimental data from which the curves are obtained are presented in Table 1.[7]

TABLE 1.

Threshold voltages (V) and threshold quantities (Q) for the durations used. Chronaxies at the beginning (1) and at the end (2) of the experiments.

Duration.	Exp. 4.		Exp. 6.		Exp. 8a.		Exp. 10.		Exp. 11.		Exp. 12.	
σ	V.	Q.	V.	Q.	V.	Q.	V.	Q.	V.	Q.	V.	Q.
3	0·173	51·90	0·123	36·90	0·102	30·20	0·168	50·40	0·112	33·60	0·200	60·00
2	0·182	36·40	0·130	26·00	0·110	22·00	0·173	34·60	0·120	24·00	0·210	42·00
1	0·212	21·40	0·164	16·40	0·138	13·80	0·205	20·50	0·149	14·90	0·241	24·10
0·8	0·228	18·24	0·180	14·40	0·150	12·00	0·218	17·44	0·163	13·04	0·260	20·80
0·6	0·255	15·30	0·201	12·06	0·170	10·20	0·240	14·40	0·185	11·10	0·289	17·34
0·4	0·303	12·12	0·246	9·84	0·206	8·24	0·285	11·40	0·227	9·08	0·340	13·60
0·2	0·452	9·04	0·368	7·36	0·301	6·02	0·400	8·00	0·340	6·80	0·498	9·96
0·1	0·715	7·15	0·528	5·28	0·438	4·38	0·580	5·80	0·537	5·37	0·770	7·70
0·08	0·790	6·32	0·600	4·80	0·505	4·04	0·630	5·04	0·607	4·85	0·877	7·01
0·06	1·030	6·18	0·690	4·14	0·610	3·66	0·780	4·68	0·735	4·41	1·050	6·30
0·04	1·150	4·60	0·855	3·42	0·750	3·00	0·960	3·84	0·895	3·58	1·330	5·32
0·02	1·920	3·84	1·020	2·04	0·950	1·90	1·350	2·70	1·230	2·46	1·820	3·64
0·01	2·580	2·58	1·220	1·22	1·160	1·16	1·580	1·58	1·480	1·48	2·200	2·20
0·005	3·800	1·90	1·440	0·72	1·330	0·66	1·840	0·92	1·600	0·80	2·320	1·16
Chronaxie												
1	0·33σ		0·46σ		0·46σ		0·33σ		0·46σ		0·30σ	
2	0·33σ		0·46σ		0·46σ		0·33σ		0·53σ		—	
Temp. C°	18·0–18·2		19·5		18·5		17·8		16·8		17·8	

The curves in Fig. 2 are drawn on a scale which makes possible the inclusion of the longest durations, so giving a survey of the whole experiment. Although showing roughly the findings concerning short durations these curves cannot represent those facts satisfactorily.

In Fig. 3 the liminal quantities for the shortest durations in the same experiments are given in detail.

[7] It might seem unnecessary to state the voltages in these Tables up to the third decimal. This, however, has been done on account of the accuracy of the voltmeter used. For example, for the range of 1·5 volt every division of the scale (150 degrees) has a value of 0·01, and as fifths of these divisions can be estimated with certainty it is clear that voltages can, in fact, be measured up to the third decimal.

With these examples in view the findings of the experiments can be described as follows: The curve representing the liminal electric quantities seems to consist of two sections: one section extends through the region of the longer durations, roughly above $0{\cdot}5\sigma$ and is, at first inspection, a straight line, crossing the abscissa somewhere above the origin; it is further discussed later. The other section belongs to the shorter durations and is a curve turning towards the origin. It is this second section which is significant for the present work. There is, so far as the experimental evidence goes, no minimum in the liminal electric quantities comparable with the intensity threshold (rheobase). These quantities decrease with decreasing duration in a progressive degree approaching zero. Whether still further reduction of the durations below the lowest value tested ($0{\cdot}005\sigma$) would lead to a lower limit is a question requiring more experimental work. It is noteworthy that all experiments without exception show the same results for the short durations[8].

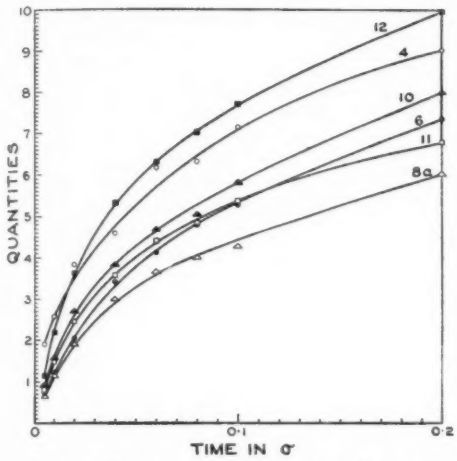

Fig. 3. The curves of Fig. 2 enlarged for durations under $0{\cdot}2$.

Turning now to the straight part of the quantity curve, this has also a bearing on the curve for short durations. If plotted on a larger scale this part of the curve is found to be really straight in only about one-third of the experiments; in the others the curve is, for durations of more than about 1σ, slightly concave upwards; in Fig. 2 are instances of both types; curves 4, 8a, 12 and also probably 11, are perfectly straight, i.e. all experimental points lie accurately in a straight line, whilst in the curves 6 and 10 the points for 2σ are slightly below, those for 3σ slightly above the straight line fitting all points best. The deviations of these two points are very slight and one might first regard them as ordinary irregularities in the distribution of experimental results. This view, however, would not be correct for two reasons: firstly, because the deviations from the straight line are in all cases of the same kind and have, consequently, to be regarded as typical or systematic, however small they may be; secondly, because there are also observations by others showing that the quantity-duration curve of the medullated nerve is, in the same region, probably slightly curved, not straight as was first believed. Lapicque (1907) was the first to claim that this curve was S-shaped, convex towards the abscissa for longer durations (above 1σ), and concave towards the abscissa for very short durations.[9] As pointed out by him (1926), Weiss has also noticed earlier some departures from the straight line of the threshold quantities.

In view of all experimental findings available, the true shape of the quantity-duration curve of the medullated nerve for durations above 1σ cannot be regarded as fully ascertained. It is beyond question that curves convex towards the abscissa occur in a considerable number of cases; but also perfectly straight quantity curves are obtained under the same conditions. It cannot be said at present whether these two results exclude each other. It seems likely that both these curves are

[8] The strength-duration curve for shortest durations should be mentioned briefly. This curve, in its ascending part, seems, with decreasing durations, to ascend from a certain point less steeply than would correspond to the course of the curve at longer durations. This change in the shape of the curve is, however, less conspicuous than the corresponding change in the quantity curve.

[9] It is this type of curve which he considered to be the only true or physiological quantity curve, corresponding to what he called a canonical strength-duration curve (Lapicque, 1931). Some of Lapicque's older experiments on the nerve have been used by Lucas (1910) to show that Hill's formula for electric stimulation could well be applied to such S-shaped quantity-duration curves. Durations under $0{\cdot}1\sigma$ were not examined.

genuine, depending on different conditions of the nerve; it is even possible that the same preparation may exhibit both curves at different times.

There is another part of the quantity-duration curve which has also to be given some attention. It is the part between $0{\cdot}5$ and 1σ. The experiments of Fig. 2 show that for these durations the curve appears to be straight, and the same is, as a rule, found for all other experiments. The results concerning longer durations can now more correctly be described as follows: for durations of more than $0{\cdot}5\sigma$ the quantity curve is either straight throughout ($\frac{1}{3}$rd of the experiments) or it is so only between about $0{\cdot}5$ and 1σ, whilst for longer durations the curve is slightly convex towards the abscissa. In such cases the whole quantity curve can be regarded as S-shaped with a short intermediary straight section between the two flexions.

Judging from the evidence obtained in the present experiments, it now appears that the true shape of the quantity-duration curve for the nerve over the usually and frequently investigated range of durations (above $0{\cdot}2\sigma$) is still somewhat uncertain and requires further investigation. The quantity curve for shortest durations, on the other hand, seems to be fairly clear.

Attention has also been given to the question as to whether the duration at which the quantity curve deviates from the straight part, and turns towards the origin, has relation to any other time value. An examination of the experiments shows that this duration is approximately of the order of the chronaxie. The points where the curves turn towards the origin can, with reasonable accuracy, be obtained from drawings made on a large scale. It appears that in about half of the experiments the difference between these times is not more than 25 p.c. of the chronaxie and only in a few experiments does this difference slightly exceed 50 p.c. of the chronaxie.

TABLE 2.

Figures for reduced quantity-duration curves.

	Experiments.						
Chronaxie.	4	5	8a	10	11	12	Mean values.
4	230·0	229·0	233·4	237·5	237·9	236·8	234·0
3·5	208·0	208·0	212·1	215·4	216·6	215·4	212·5
3	185·8	186·3	189·7	195·2	193·8	192·3	190·3
2·5	163·7	165·0	168·4	170·1	170·7	176·6	169·1
2	143·4	128·4	147·8	147·2	148·4	147·9	143·8
1·5	121·4	110·0	135·3	123·3	124·1	123·4	122·9
1	100	100	100	100	100	100	100
0·8	91·5	91·1	88·7	88·4	89·2	88	89·5
0·6	82·3	81·9	78·5	76·0	78·6	81·2	79·7
0·4	72·6	71·9	65·1	62·5	67·2	70·0	68·2
0·2	53·1	52·7	48·3	45·7	52·5	63·9	51·3
0·1	39·8	44·4	36·4	32·2	39·7	39·3	38·6
0·05	29·6	28·6	23·0	22·1	20·7	25·6	24·9
0·02	23·8	13·1	14·0	15·5	18·0	14·5	15·5

The actual numerical results obtained from the experiments differ considerably and so do, necessarily, the quantity-duration curves. It is desirable, however, to compare the individual experiments with each other and even with the experiments made with condenser discharges. This can be done if the curves are brought to biologically equivalent units, taking the chronaxie in each case as the unit for the durations and the quantity for chronaxie stimulation as the unit for the threshold quantities. The quantities for different fractions and multiples of the chronaxie are best obtained from curves drawn on a very large scale. If the actual figures are transformed according to these units, new curves on a uniform scale are obtained which can be called "reduced" quantity curves. The reduced figures for the six experiments presented in Fig. 2 and Table 1 are shown in the Table 2. It

can be seen that these figures, in spite of the differences in the actual experimental values, agree well with each other. A curve drawn from the mean values of the reduced curves can be regarded as a concise representation of the typical quantity curve for stimulation with galvanic currents. The curve "A" in Fig. 4 is the mean reduced curve of the six curves. The main features of this curve are: for durations below the chronaxie the curve is concave towards the abscissa, for durations 1 to 4 times the chronaxie the curve is straight. These two parts meet approximately at a duration equalling the chronaxie.

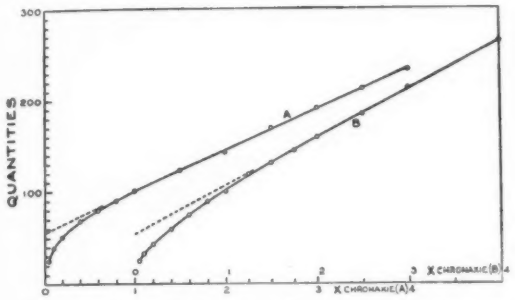

Fig. 4. Reduced quantity-duration curves, A = for stimulation with galvanic currents, B = for stimulation with condenser discharges. For explanation see text.

(b) *Experiments with Condenser Discharges.*

Stimulation with condenser discharges yields results similar to those obtained with direct currents, the capacities being used to determine the duration of the electric shocks. As shown by Hoorweg long ago, there is a characteristic relationship between various capacities and the threshold voltages, i.e. the voltages to which the capacities have to be charged to give liminal stimulating effects. These voltages plotted against the capacities fall upon a curve (voltage-capacity curve) which is analogous to the strength-duration curve. The liminal electric quantities lie very nearly on a straight line.

Accordingly the excitability can also be studied by using condenser discharges as stimuli. The chronaxie can in this way be determined by means of a method worked out by Lapicque (1926).[10] Some additional experiments with condensers were also made for the present work.

TABLE 3.

Capacities microfarads.	Durations of equivalent direct currents. σ	Capacities microfarads.	Durations of equivalent direct currents. σ
0·4	4·73	0·02	0·24
0·3	3·55	0·01	0·12
0·2	2·37	0·008	0·095
0·1	1·18	0·006	0·071
0·08	0·95	0·004	0·047
0·06	0·71	0·002	0·024
0·04	0·47	0·001	0·012

These experiments were arranged similarly to those with galvanic currents; the liminal charges to a series of suitable capacities were determined, proceeding gradually towards the higher capacities or vice versa. To give an idea of the approximate order of the time values represented by the capacities used, the durations of equivalent direct currents, calculated from Lapicque's formula[11] are stated in Table 3 for the resistance used in the experiments mentioned below. It can be seen that the capacities roughly cover the same field of durations as the galvanic currents.

Twenty-five experiments with condensers were made. The results, which agree in all essentials with the results described above, can be dealt with briefly.

[10] According to his experiments the chronaxie, as defined for direct currents, can be calculated approximately from the formula: $CR \times 0{\cdot}37$, where C = capacity (farad) which if charged to a voltage twice the rheobase will give liminal effects, R = resistance (ohm.) and 0·37 an empirical factor. The magnitude of this factor has been confirmed by others and is frequently used; other observers found different values, e.g. Vogel (1925) obtained the factor 0·51.

[11] The value of such calculations will depend on the correctness of the above-mentioned factor. Whether Lapicque's factor applies to all irritable tissues and to the whole range of capacities is uncertain and requires more investigation.

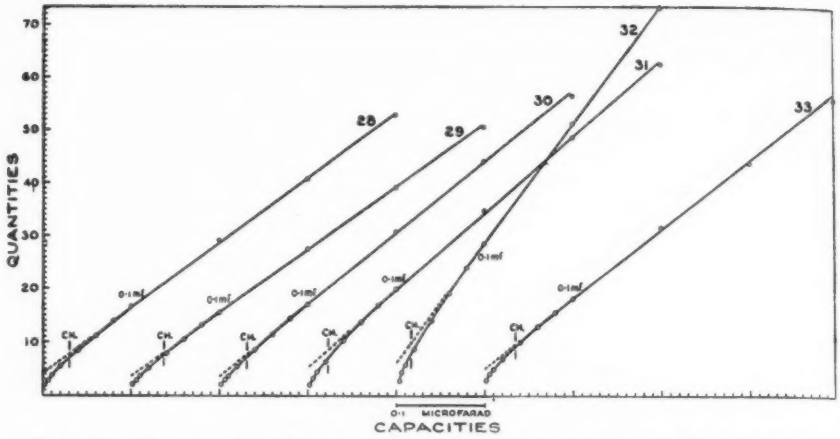

Fig. 5. Quantity-capacity of six experiments. Ch = chronaxie capacity. Compare Table 4.

In Fig. 5 are presented 6 typical quantity curves. The similarity with the curves for galvanic stimulation is obvious. Here again the liminal quantities for greater capacities (above 0·1 mf.) fall roughly upon a straight line whilst for smaller capacities they turn towards the origin. The experimental figures are presented in Table 4. According to these findings there is no lower limit for the effective electric quantities of condenser discharges; as in the case of galvanic stimulation the threshold quantities decrease progressively with decreasing capacities. In the present experiments the quantities have not been investigated quite so far downwards as in the case of galvanic stimulation, the smallest precision condenser available being one of 0·001 mf. Nothing can be said as to still smaller capacities. It might be mentioned here, that in some recorded experiments with condenser stimulation the same turn towards the origin of the liminal quantities can be noticed (e.g. Bourguignon, 1923); but this fact has remained uninvestigated or even unnoticed.

TABLE 4.

Threshold voltages (V) and threshold quantities (Q) for the capacities use. Chronaxies at the beginning (1) and at the end (2) of the experiments.

Capacities.	Exp. 28.		Exp. 29.		Exp. 30.		Exp. 31.		Exp. 32.		Exp. 33.	
μF.	V.	Q.	V.	Q.	V.	Q.	V.	Q.	V.	Q.	V.	Q.
0·4	1·320	5·28	1·265	50·60	1·400	56·00	1·560	62·40	2·360	94·40	1·400	56·00
0·3	1·360	4·08	1·305	39·15	1·472	44·16	1·620	48·60	2·440	73·20	1·460	43·80
0·2	1·460	2·92	1·380	27·60	1·540	30·80	1·740	34·80	2·560	51·20	1·580	31·60
0·1	1·660	1·66	1·560	15·60	1·712	17·12	2·000	20·00	2·860	28·60	1·820	18·20
0·08	1·720	13·76	1·640	13·12	1·800	14·40	2·120	16·96	3·000	24·00	1·960	15·68
0·06	1·860	11·16	1·780	10·68	1·940	11·64	2·300	13·80	3·180	19·08	2·160	12·96
0·04	2·100	8·40	2·000	8·00	2·180	8·72	2·600	10·40	3·520	14·08	2·520	10·08
0·02	2·760	5·52	2·640	5·28	2·840	5·68	3·280	6·56	4·400	8·80	3·480	69·60
0·01	3·780	3·78	3·720	3·72	3·880	3·88	4·340	4·34	5·800	5·80	5·080	5·08
0·008	4·260	3·40	4·200	3·36	4·400	3·52	4·800	3·84	6·500	5·20	5·880	4·70
0·006	4·600	2·76	4·920	2·95	5·160	3·09	5·480	3·28	7·400	4·44	7·000	4·20
0·004	6·400	2·56	6·400	2·56	6·640	2·65	6·800	2·72	9·200	3·68	9·200	3·68
0·002	10·000	2·00	10·400	2·08	10·500	2·10	10·100	2·02	14·000	2·80	15·000	3·00
0·001	—	—	17·800	1·78	17·500	1·75	15·800	1·58	—	—	—	—
Chronaxie												
1	0·030 μF		0·037 μF		0·030 μF		0·025 μF		0·012 μF		0·040 μF	
2	0·030 μF		0·038 μF		0·032 μF		0·019 μF		0·019 μF		0·030 μF	
Temp.C°	16·6–17·3		17·4–17·1		17·5–17·3		17·6–17·8		17·2		18·7–18·8	

Closer examination of the results, and comparison with those of galvanic stimulation, reveals some differences which must be taken into consideration; these differences refer to the smaller as well as to the greater times or capacities.

(a) Inspection of the curves in Fig. 5 shows that the regions for smaller capacities are flatter than the corresponding ones for galvanic stimulation. For better comparison the reduced quantity curves must be examined as presented for this purpose side by side in Fig 4. How these curves are obtained has been explained; the only difference in the procedure is that for condenser stimulation capacities replace the durations, the unit being the chronaxie capacity, and that the unit for the quantities is the quantity for chronaxie stimulation.

TABLE 5.

Figures for reduced capacity-duration curves.

Chronaxie capacity.	Experiments 28	29	30	31	32	33	Mean values.
4	268·5	295·0	269·5	251·9	260·4	243·4	264·8
3·5	241·4	263·1	241·3	228·5	235·7	219·2	238·6
3	215·7	235·5	215·7	207·1	210·9	196·9	213·6
2·5	188·5	203·6	170·2	185·7	186·1	171·1	184·3
2	159·4	173·4	158·8	159·0	156·6	150·2	159·6
1·75	145·7	156·8	143·2	148·5	142·7	137·8	145·8
1·5	130·7	141·3	129·3	132·0	128·9	125·2	131·2
1·25	125·7	124·4	114·4	120·0	114·7	113·2	118·7
1	100	100	100	100	100	100	100
0·8	87·8	93·0	86·8	86·4	87·2	87·4	88·1
0·6	77·1	78·2	73·0	72·1	74·2	77·3	75·3
0·4	59·7	62·9	58·4	57·8	59·9	60·9	59·6
0·2	41·4	45·0	41·8	41·4	43·2	45·9	43·1
0·1	31·4	33·9	32·4	30·0	35·2	36·1	33·2
0·05	—	27·7	26·1	22·8	—	—	25·5
0·025	—	22·8	—	—	—	—	—

Table 5 gives the reduced threshold quantities of the 6 experiments in Fig. 5, the values, as in the case of galvanic stimulation, showing very good agreement. The two reduced curves of Fig. 4 demonstrate best the difference in the course of the quantities for times or capacities below the chronaxie value.

(b) The reduced curves show another difference between the results of the two methods. For galvanic stimulation the duration at which the liminal quantities deviate from the straight part of the curve, and turn towards the origin, is, in most cases, within 50 p.c. of the chronaxie; on the other hand the capacities at which the same occurs for the quantities of condenser discharges differ much more from the chronaxie capacity. This can be seen in the 6 examples of Fig. 5.

In a considerable proportion of the experiments the threshold quantities leave the straight line at capacities 2 to 3 times greater than the chronaxie capacity. In the mean reduced curve derived from the 6 examples this point coincides with a capacity twice the chronaxie value. In only one-fourth of the experiments does this critical capacity differ from the chronaxie capacity by less than 50 p.c.

(c) The shape of the quantity curve for capacities above the chronaxie differs also from the corresponding part of the curve for direct current stimulation. For capacities 2 to 4 times greater than the chronaxie the curve, in most cases, appears to be straight but for greater capacities the curves exhibit some slight concavity towards the abscissa. This is noticeable in Fig. 5 if the deviation of the experimental points from the nearest possible straight line is considered. In such cases the whole quantity curve can be regarded as consisting of two parts concave towards the abscissa, one with a stronger curvature at the lower capacities the other one with a slight curvature at the larger capacities; between these two parts there seems to be a short straight part.

Only about one-third of the quantity curves are completely straight above the critical capacity. The upward concavity, frequently occurring with galvanic stimulation, was not encountered. It must be assumed that these differences are due to differences in the stimuli. As to the main problem here in question, the results for small capacities agree essentially with the corresponding results for short galvanic currents.

REMARKS.

The main conclusion from the present work is that the liminal electric quantities decrease from a certain point in a progressing degree; consequently, the corresponding part of the quantity-duration curve presents a strong concavity to-

wards the abscissa. It is not within the intended scope of this paper to examine whether the data obtained for currents of very short duration can satisfactorily be expressed by any formula. The following comments are, however, relevant.

The apparent discrepancy between curves obtained with rectangular waves and with condenser discharges (Fig. 4) seems to depend on the manner in which the stimulus becomes effective. For liminal galvanic currents the relative values of the effective quantities can easily be determined. This applies also to condenser discharges; but as it is the initial part of the discharge that produces the full effect, the actual quantity passed into the tissue is not equivalent to the effective quantity. To meet this difficulty it will be necessary to determine the actually utilized part of the discharge, i.e., to determine the "utilization time"[12]; but experiments on this have had to be postponed. It may perhaps be possible to express the condenser discharges as equivalent galvanic currents, using Lapicque's formula (p. 163) for this conversion; but this would necessitate a preliminary investigation on the validity of applying Lapicque's factor (0·37) to short duration.

Turning now to the curve for short durations, it would seem hardly likely that this could be considered independently of the curve for longer durations. Can the entire curve be represented by one single equation; and, if so, are any of the existing formulæ, derived from results with longer duration, applicable? In Fig. 6 the quantity-duration curves for the four most discussed formulæ are placed beside the actual experimental quantity curve. The shaded areas mark the shortest durations. It will at once be seen that Weiss's curve fails entirely for short durations, but applies for the longer durations when, as often, these lie on a straight line. Nernst's curve, while applying to short durations, does not fit well for long durations. Both Lapicque's and Hill's formulæ give S-shaped curves, representing more or less satisfactorily the quantities at longer durations, and they might also conform with the quantities at short durations, Hill's formula apparently giving the closer fit. But the validity of these, or of any other formula, for the whole range of durations will depend, in large measure, not only on the form of the curve for short duration, but also on that for long durations, the actual form of the latter, i.e. whether straight or concave towards the abscissa, being not yet known with certainty.

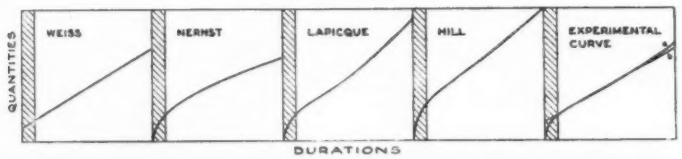

Fig. 6. Quantity-duration curves according to the equation of Weiss, Nernst, Lapicque and Hill and the quantity-duration curves found in the experiments, a = concave upwards, b = straight. Shaded areas mark the very short durations.

To what extent the equation for electric stimulation really accords with the results described for shortest durations will be dealt with in another paper.

It should finally be pointed out that the above results have to be supplemented by an investigation of the changes which occur in the nerve at the cathode (cathode potential) when passed by currents of such short duration.

[12] The "utilization time" of condenser discharges has already been investigated, e.g. by Lapicque (1911).

SUMMARY.

Nerve stimulation with electric currents of very short duration has been investigated using as stimuli both galvanic currents (rectangular waves), and condenser discharges.

The results of both methods are essentially the same, namely that at durations under 0·2 milliseconds or for capacities under 0·1 micro-farad the electric quantities required for minimal stimulation decrease with decreasing duration (capacity) in a progressive degree. Accordingly, the corresponding part of the quantity-duration curve shows a strong concavity towards the abscissa. No lower limit (threshold) for the effective electric quantities could be found within the range examined.

Some features of the stimulation curve for longer duration are also considered.

A preliminary discussion is given of the relation of the results obtained to some of the most important formulæ for electric stimulation.

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THE EFFECT OF THE TEMPERATURE OF PRELIMINARY INCUBATION ON SUSCEPTIBILITY OF THE CHICK EMBRYO TO INFLUENZA VIRUSES

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The fact that the chick embryo is more susceptible to influenza virus when incubation *after* inoculation is below the physiological normal, for instance at 36° C., was first observed by Burnet (1936) and later confirmed by Miller (1944) and Sigurdssen (1944). However, there has been no report indicating that the temperature of incubation *before* inoculation has any influence on the susceptibility of the embryos to any virus or on the amount of virus produced.

In his earlier work Burnet (1936) used for preliminary incubation an old type of still-air incubator at 39·5° C. (103° F.) and in work reported in 1938 used a modern electrical forced-draught incubator at 37·8–38·3° C. (100–101° F.). Influenza infection of the allantoic cavity has recently been studied by Sigurdssen (1944) and Knight (1944) in eggs previously incubated at 38·5° C. and 39° C. respectively, and by Miller (1944) in eggs, some of which had been incubated at 37° C. and some at 39° C. Most authors do not state the temperature of preliminary incubation.

EFFECT OF TEMPERATURE OF PRELIMINARY INCUBATION ON ALLANTOIC INFECTION.

While producing allantoic fluid virus for vaccine purposes we found that when eggs had been incubated in a forced-draught incubator at 38·6° C. before inoculation they yielded small volumes and a proportion of them failed to become infected as judged by the haemagglutination test. One hundred and eleven eggs inoculated at 10 days with influenza virus A, strain BEL, 41st embryo passage, yielded an average of 5 ml. and 113 inoculated with influenza virus B, strain BON, 39th embryo passage, yielded an average of 3·5 ml. per egg. The temperature of the incubator was then altered to 37·5° C. after which the volume of the allantoic fluid obtained increased and the proportion of eggs failing to give haemagglutination decreased. One hundred and ten eggs inoculated at 10 days with the A strain yielded an average of 8 ml. and 71 eggs inoculated with the B strain yielded an average of 7·5 ml. The temperature of incubation after inoculation was 35° C. in all cases. These eggs had an average weight of about 69 gm. before incubation.

Five set experiments were then carried out using for each experiment eggs from the same batch treated at the same time and in the same manner except that the temperature of incubation before inoculation differed in each group. The relative humidity during this preliminary inoculation was approximately 65 p.c. The strains of virus used had had 39 or 41 passages in embryos. They were inoculated at a dilution of 10^{-2} of allantoic fluid virus, that is about 10^4 infective doses in eggs previously incubated at 37° C. All eggs were incubated at 35° C. for 2 days after inoculation. They were then chilled and the allantoic fluid taken off, tested for haemagglutination at dilutions of 1/5 and 1/50 and the volume measured. The details of the experiments and the results are set out in Table 1. Eggs in all groups except the last one in experiment III were inoculated at 10 days.

The results of experiments I, II and III show that eggs incubated at 39° C. as compared with those incubated at 37° C. yield only about half as much allantoic fluid and are much less susceptible to infection. This effect is not offset by holding the eggs at 35° C. for 2 or 3 hours before inoculation. The volumes of fluid in the eggs which did not become infected were about the same as the infected eggs in the same groups. The results of experiments IV and V suggest that eggs incubated at 36·6° C. may yield slightly larger volumes and be slightly more susceptible than eggs incubated at 37·8° C. but there are too few in the groups to place reliance on these slight differences. By susceptibility is meant the proportion of embryos which show evidence of infection after inoculation with a given dose by giving haemagglutination at 1: 5.

The slight difference in the results after 39° C. in experiments I and III may have been due to the fact that in experiment I the eggs were inoculated after being only a few minutes at room temperature whereas in experiment III they were left at room temperature half an hour before inoculation.

Judging by a rather limited series of haemagglutination titrations, the titres of the positive fluids from the eggs incubated at 39° C. tended to be rather higher than from those incubated at 37° C. or 37·8° C., but the differences were not sufficient to compensate for the differences in volumes yielded. Most allantoic fluids from all groups either gave no haemagglutination at 1/5 or else were strongly positive at both 1/5 and 1/50 dilutions. Five allantoic fluids which gave no haemagglutination at 1/5 were sub-inoculated into further eggs; all gave positive fluids in the sub-inoculated eggs. The titre of a pool of all the fluids from the group of 11 day embryos in experiment III was only about half as high as the titres of the two pools from the two groups of 10 day embryos.

TABLE 1.

Allantoic inoculation of influenza viruses in embryos incubated at different temperatures.

Experiment.	Temperature before inoculation.	Strain.	Eggs. Positive haemagglutination at 1: 5.	Eggs. Negative haemagglutination at 1: 5.	Average volume of allantoic fluid in positive eggs.
I	37° C.	BON	39	0	7 ml.
	39° C.	BON	6	6	2 ml.
	39° C., last 2 hrs. 35° C.	BON	7	5	3·5 ml.
II	37° C.	BEL	23	0	7 ml.
	39° C.	BEL	11	4	4·5 ml.
	39° C., last 3 hrs. 35° C.	BEL	12	3	4·5 ml.
III	37° C.	BON	25	0	7·6 ml.
	39° C.	BON	12	4	5·2 ml.
	37° C., (11 days)	BON	24	0	10·1 ml.
IV	36·6° C.	BEL	11	0	8·2 ml.
	37·8° C.	BEL	11	1	7·8 ml.
V	36·6° C.	LEE	9	0	8·2 ml.
	37·8° C.	LEE	13	0	7·2 ml.

The eggs were inoculated after 10 days incubation at the temperature stated, except the last group in experiment III which was incubated 11 days before inoculation. All eggs were inoculated with allantoic fluid virus diluted 10^{-8}, that is, about 10^4 infective doses in eggs previously incubated at 37° C., and then were incubated 2 days at 35° C.

The results obtained in experiment III and our general experience show that the differences in susceptibility and in volume of allantoic fluid in eggs incubated at 39° C. or 37° C., are not due to differences in maturity of the embryo.

Each egg in experiment III was weighed before incubation and again before inoculation. The average loss of weight was 3·3 gm. at 37° C. and 3·6 gm. at 39° C. Therefore, the difference in volume of allantoic fluid after 39° C. or 37° C. is not due to greater evaporation from the eggs at the higher temperature. In eggs inoculated after incubation at 39° C. and showing very little allantoic fluid, the yolk sac is quite flaccid and appears to contain a large amount of watery yolk; in eggs incubated at 37° C. or 37·8° C. before inoculation the yolk sac and contents are less voluminous and have an almost doughlike consistence. Probably after incubation at 39° C. the bulk of the water is in the yolk sac and after 37° C. is in the allantoic cavity, but we have not measured any yolk volumes.

Some experiments were also carried out with influenza virus B in relatively unadapted form, the third amniotic passage of BON. As with the adapted strains, eggs incubated at 39° C. until inoculation were less susceptible than those incubated at 37° C. Eggs incubated at 39° C. until 2 days before inoculation and then at 37° C. gave the same results as eggs incubated at 37° C. during all the preliminary incubation period. The results obtained with some small groups suggested that embryos incubated at 32° C. or 35° C. during the last 2 days before inoculation may be slightly more susceptible than those incubated at 37° C.

EFFECT OF TEMPERATURE OF PRELIMINARY INCUBATION ON INFECTION BY OTHER ROUTES.

Two strains of influenza virus were available which were only one and two passages removed from the original human material. First yolk sac passage of an influenza A strain and second amniotic passage of BON were titrated by amniotic inoculation in eggs incubated at 39° C. and in eggs incubated at 37° C. before inoculation. There was no difference at all between the two groups in susceptibility as judged by the minimum dose which produced infection in any of the eggs or the proportion of embryos infected at each dilution. First yolk sac passage of influenza A was titrated by yolk sac inoculation in 6 day eggs incubated at 39° C. and 37° C. There was no difference between the two groups.

The growth of ectromelia virus on the chorioallantois was reported by Burnet (1938) to be affected by the temperature of incubation after inoculation and by changes in the eggs due to unknown factors apparently associated with season. It was, therefore, chosen as a suitable virus with which to test the susceptibility of the chorioallantois after incubation at 39° C. and at 37° C. Eggs, incubated at 39° C. or at 37° C. before inoculation showed no significant difference in the number of foci or size of foci, there being considerable variation within each group in both respects. However, in this series 17 membranes of 32 in the 39° C. groups showed bad non-specific scarring whereas only 3 membranes of 41 did in the 37° C. groups. This scarring follows haemorrhage which occurs when the chorioallantois is dropped (Burnet and Faris, 1942). There was no difference between the two groups in the numbers of membranes showing non-specific oedema or slight diffuse opacity.

DISCUSSION.

Contrary to our early hopes, the temperature before inoculation does not influence the susceptibility of all tissues of the embryo to viruses. There is a marked effect on the susceptibility to influenza virus of the cells lining the allantoic cavity, but there is no appreciable effect on susceptibility to influenza inoculated into the amniotic cavity or the yolk sac nor to ectromelia virus inoculated on the chorio-allantois.

Allantoic inoculation of embryos is now used extensively for titrating influenza virus in connection with various investigations, often requiring a considerable degree of accuracy. The effect of the temperature of preliminary incubation of the eggs has been an unsuspected possible source of error in this work. Some workers record that the eggs they used had been incubated at a temperature which we have shown leads to irregular results, although most authors do not state the temperature of preliminary incubation. Miller (1944) commented on what he called the inherent variation of different batches of eggs in resistance and in the volume of allantoic fluid influenza virus produced. Probably these differences were due to the different temperatures of preliminary incubation which he used. Burnet and Faris (1942) reported that the liability of embryos to have haemorrhages when the chorioallantois is dropped, and hence non-specific lesions, varied from season to season and sometimes from week to week. In view of the observations reported here, these variations were probably due to small fluctuations in the temperature of incubation before inoculation.

In the old type of still-air incubator the optimum temperature of incubation for hatching is usually regarded as 39·5° C., but this is read just above the egg which is itself at about 1° C. lower temperature. In the forced-draught type of incubator the optimum temperature for hatching is 37·8° C. (Barott, 1937).

SUMMARY.

Embryos incubated at 39°C. (102° F.) before inoculation differ from those incubated at 36·6–37·8° C. (98–100° F.) in the following respects: (i) they yield only about half the volume of allantoic fluid after inoculation with influenza virus, (ii) a proportion of them are not infected, as judged by haemagglutination, by an inoculum of influenza virus A or B which produces uniform infection in em-

bryos which have been incubated at the lower temperature, (iii) when the chorioallantois is used for inoculation bad scarring more often occurs. The temperature of incubation before inoculation has no influence on the susceptibility to influenza virus A or B inoculated into the amniotic cavity or the yolk sac, nor to ectromelia virus inoculated on the chorioallantois.

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RE-EXAMINATION OF THE INFLUENZA VIRUS STRAIN "MELBOURNE EGG"

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In 1935-36 a strain of influenza virus isolated from a patient in Melbourne during July, 1935, was adapted to growth on the chorioallantois (Burnet, 1936). After 60–70 passages the strain subsequently referred to as "Melbourne Egg" or M.E. had the following characteristics: On the chorioallantois it produced small but distinct focal lesions whose number was closely proportional to the concentration used, blood stream infection of the embryo followed the initiation of chorioallantoic lesions, and death resulted usually between 48 and 72 hours. Post mortem there were numerous haemorrhages in skin, muscles and brain which gave an unmistakable picture of specific death. The virus was almost avirulent for ferrets but infection was followed by active immunity to a virulent strain and a normally high antibody titre.

The strain was used for a large number of studies on influenza antibodies and for some unsuccessful attempts at human immunization during the period 1936–40 (Burnet and Lush, 1938). With the development of amniotic and allantoic cavity methods of inoculation and Hirst's haemagglutination technique much more convenient methods of study became available and interest in the chorioallantoic studies lapsed almost entirely. We have recently been stimulated to re-examine the strain in the light of more modern methods primarily on account of the fact that alone amongst all the stock strains of influenza virus in our possession it had failed to show any agglutination of fowl red cells and grew very poorly in the allantoic cavity.

Experiments were commenced with material which had undergone successively 5 ferret passages, 103 chorioallantoic, 2 amniotic and 17 chorioallantoic passages. Seventieth passage material dried in 1936 was recovered in September, 1938, and maintained by infrequent passage with intermediate storage in glycerol-Ringer at — 10° C. till March, 1940. At this stage a "single pock" reisolation of the strain was made. Only two passages were made in 1940 and in 1941 amniotic fluid virus was prepared for storage in a dry ice refrigerator. A further 9 passages from this gave material which was similarly stored in June, 1943. The strain was revived in December, 1943, and given 8 chorioallantoic passages before the series of amniotic inoculations to be reported was commenced.

Early in 1944, one of us (D.R.B.) observed that although allantoic inoculation almost always killed the embryo the allantoic fluids contained no agglutinin for fowl cells. A few fluids, particularly from eggs inoculated amniotically showed a rather atypical agglutination of the embryonic red cells which were present in them. Direct examination showed that such fluids could produce agglutination of red cells from embryo chicks but not from adult fowls.

To examine this phenomenon in detail several embryos were inoculated amniotically with pooled amniotic fluid (first passage after 103 + 17 chorioallantoic passages). These embryos gave after 2 days incubation turbid amniotic fluids. On titrating one of these with both adult and embryonic chick cells it was found that although the embryonic cells gave a higher titre there was distinct agglutination of the adult fowl cells. This agglutination was of poor quality and quite strikingly resembled the O agglutination of an unadapted influenza virus (Burnet and Bull, 1943). A test with guinea-pig and human red cells was therefore made giving the very unexpected finding that M.E. possessed the same agglutinin activity as a freshly isolated "human" O strain of virus, the F/G ratio in the first instance

[1] This work was supported by a grant from the National Health and Medical Research Council of the Commonwealth Government.

examined being 30/240. A further resemblance to the O strains is the irregularity of infection by the allantoic route. This had been casually noted on several previous occasions and was one of the main reasons for discarding the strain when the new methods were developed.

TECHNIQUE.

In applying the Hirst haemagglutination technique to problems with M.E. two undescribed techniques have proved useful. (1) Spontaneous agglutination of the embryo blood cells in chicks dying after intravenous infection can be demonstrated with regularity by using a fine pointed capillary pipette to remove a small quantity of blood from the distended allantoic veins as it enters the body stalk. The pipette is washed out into a suitable volume of saline to give an appropriate density of red cells. The cells are then allowed to sediment in the usual manner and show typical O type agglutination. Experiments demonstrating the high virus concentration of the blood will be described later. (2) When the whole of the allantoic fluid is removed from an embryo which has been inoculated allantoically with M.E. it is possible to use the haemagglutinin test to measure the virus concentration even when undiluted fluid shows no agglutination by the standard technique with one volume of fluid (0·25 ml.) one of saline and one of red cells. This depends on the fact that if a small volume of red cells is allowed to sediment slowly through a column of dilute virus the cells will pick up enough to allow their agglutination by the time they are deposited on the bottom of the tube.

The set-up we have used for measuring very small amounts of M.E. agglutinin in allantoic fluids is as shown:

Allantoic fluid	2	1	0·5	0·25
Saline	—	—	—	0·25
Red cells	0·25	0·25	0·25	0·25
"Dilution"	0·125	0·25	0·5	1

Guinea-pig red cells are used and owing to the long time required for these to deposit it is most convenient to leave the tubes overnight in the refrigerator before reading. Patterns produced by the deposition of guinea-pig cells on the bottom of a tube show very little tendency to slip or blur in the refrigerator. Using this technique it is possible to titrate fluids with titres in the range 0·1 to 1,000 or more.

Intravenous inoculation of chick embryos was carried out by a technique kindly demonstrated to one of us by Dr. C. A. Brandly. It depends on the clarification of the shell membrane over a suitable chorioallantoic vein by a drop of sterile liquid paraffin. The inoculation is made with a fine needle using a constant volume of 0·1 ml. of inoculum.

All the other technical methods used have been frequently described from this laboratory or elsewhere.

Comparative Titration of M.E. by Different Methods.

The methods of titration available are limited to haemagglutination and those involving inoculation into chick embryos by various routes, since the strain is avirulent for mice and ferrets. It was of particular interest to compare what was previously used as the normal method of titration, enumeration of foci on the chorioallantois, with the more recently developed methods of amniotic allantoic and intravenous inoculation in chick embryos.

TABLE 1.

Comparative titration of amniotic fluid 89059 of haemagglutinin titre F/G 40/200.

Titration method and volume of inoculum used.		Virus dilutions. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Chorioallantoic	0·05 ml.					9·5	0·6	0	
Allantoic	0·05 ml.	3/4	4/4	3/4	4/4	3/4	1/4		
Amniotic	01 ml.					4/4	3/3	0/4	
Intravenous	0·1 ml.						5/5	4/5	1/5

Chorioallantoic results = mean count of foci. Others show proportion of embryos showing positive results either by agglutination of guinea-pig red cells or by specific death of the embryo.

TABLE 2.

Combined data of three comparative titrations.

Preparation.	Focal count per ml.	50 p.c. Infective doses per ml. Intravenous.	Amniotic.	Allantoic.	Haemagglutinin titre F/G.
88815 Amn.	$10^{7\cdot7}$	$10^{8\cdot6}$			240/800
88819 All.	$10^{7\cdot0}$			$10^{3}\pm$	12/70
89059 Amn.	$10^{7\cdot3}$	$10^{8\cdot5}$	$10^{8\cdot5}$	$10^{8\cdot6}$	40/200

Three such comparative titrations were carried out. Table 1 gives the detailed results of the most comprehensive test while Table 2 combines the results from the three experiments.

It will be seen from Table 2 that amniotic and intravenous methods are of approximately equal sensitivity but that the chorioallantoic focal count represents only about 1/5–1/10 of the minimal number of infective particles which must be present in the preparation.

The allantoic titrations showed frequent irregularities and it seems worth while to present the results of that given in Table 1 in further detail to illustrate the frequent dissociation of positive haemagglutinin findings and embryo death. In this experiment as in most of those we have made with this strain, repeated tests of allantoic fluid for agglutinin have been made on consecutive days. On the 3rd day embryos showing positive agglutination were examined for lesions. All the remaining eggs were reincubated for another day and then finally examined for both haemagglutinin and embryonic lesions. Table 3 shows the day after inoculation on which haemagglutination was first observed and on which the embryo died with specific lesions.

TABLE 3.

Result of allantoic titration of M.E. amniotic fluid 89059.

Indication of virus activity.	Virus dilution. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Specific death of embryo	33 –3	3433	434–	– 433	4 – – –	4 – – –
Haemagglutination	22 –2	– – 22	– 2 – –	3333	444 –	– – – –

Days on which positive indication was obtained. Results for each embryo shown in same order.

It will be seen that in the lower dilutions embryos which showed no haemagglutination on the second day did not develop it later though several died with specific lesions.

PATHOGENCITY FOR FERRETS AND MICE.

Two ferrets F191 and F192 were inoculated intranasally with the chorioallantoic membrane emulsion used to initiate the current series of experiments (88256–61). No symptoms or temperature rise resulted, the maximum temperatures reached being 102·6° F. and 102° F. respectively. Two days after inoculation the noses of the two animals were washed out with 15 c.c. of broth according to the method previously described (Burnet and Bull, 1943). The washings were filtered through gradocol membranes and titrated on the chorioallantois. F191 washings gave 10, 6, 6, 4 foci from undiluted filtrate, F192 2, 1, 0, 0. In both animals, therefore, there is adequate evidence that infection occurred despite the absence of significant temperature rise. Blood samples were taken before inoculation and 14 days afterwards. In an attempt to induce a higher antibody titre both were reinoculated subcutaneously with high titre amniotic fluid virus (M.E.). The first bleedings gave sera with unusually high inhibitory titres in haemagglutination tests but there was a typical high specific titre when the second or third blood samples were tested against M.E. using guinea-pig cells.

The titres were: F191 I (normal), 280; II, after intranasal infection, 2,400; III, after subcutaneous inoculation, 2,400. F192 I, 280; II, 1,300; III, 2,400. Further serological reactions of these sera are described later. For the present this is sufficient to establish the fact that in ferrets the strain behaves as it did when tested 8 years previously, producing a subclinical infection with an effective immune response.

In mice undiluted allantoic fluid of moderate haemagglutinating titre has no capacity whatever to produce pulmonary lesions. In previous experiments (Burnet, 1937) M.E. was shown to be an excellent immunizing agent for mice against the homologous mouse adapted strain. This has been confirmed for the strain in its present condition. Results are now, however, more striking since the strain is totally without power to provoke mouse lung lesions even when much more concentrated material than was previously available is used.

TABLE 4.

Immunization of mice by M.E. virus given intranasally.

Dose of M.E. used for immunization.	Test dilutions of "Melbourne" (mouse) allantoic fluid. 10^{-1}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
M.E. undiluted 0·05 ml.	1, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0			
M.E. 1: 100 0·05 ml.	0, 0, 0, 0, 0, 0	47, 0, 0, 0, 0, 0			
Controls			3 + 3 + 3 + 3	3, 2+, 2, 2	2, 1, 1, 0
	Immunizing virus	M.E. 89449	Titre F/G 6/32		
	Challenge virus	M.M. 90018	Titre F/G 240/140		

Lung lesions 7 days after challenge inoculation are shown according to the usual convention.

Table 4 shows the result when challenge tests were made with active Melbourne mouse virus 2 weeks after intranasal inoculation with M.E. Except for one animal which possibly failed to take the immunizing dose into the lung, immunity is absolute against up to 10^4 infecting doses of mouse virus.

A few of the immunized mice were tested for resistance to the strain W.S. Of 7 immunized with either undiluted or 1: 100 M.E. all survived a test dose of 100–1,000 lethal doses, four with no areas of consolidation. The other three had extensive consolidation.

ANTIGENIC DIFFERENCE BETWEEN EGG AND MOUSE PASSAGE STRAINS OF MELBOURNE.

The strain Melbourne has also been carried since 1935 by mouse passage and in 1941 a substrain was taken through a series of amniotic and allantoic passages. The current "Melbourne mouse" (M.M.) strain is derived from a stored dried stock of 50th amniotic passage material and is used in the form of allantoic fluid. Such infected fluids give a haemagglutination titre of the type F/G 480/220 and will infect mice to a titre of about 10^6 (average 2 + lesion) killing at 10^4 and below.

Sera from several ferrets inoculated with strain M.M. were available. One of these F83 was used in comparison with two sera made against the current M.E. strain F191 and F192.

Haemagglutination tests with M.E. were made using guinea-pig cells, while the more convenient fowl cells were used with M.M. Allantoic cavity experiments were made according to the usual technique but with M.E. the fluid was sampled at 3 days and the eggs then returned to the incubator for 2 days when they were opened and the embryos examined for specific lesions.

Haemagglutination tests showed a consistently greater neutralizing power of the homologous ferret sera. In Table 5 are shown the titres of the three sera against the two types of virus the serum F83 being given the value 1·0 with virus M.M. and F191 being taken as the standard against M.E.

TABLE 5.

Haemagglutinin titration of ferret immune sera.

Sera.	Against M.M.				Average.	Against M.E.				Average.
F 83 (M.M.)	1	1	1	1	1	0·5	0·34	0·18	0·16	0·29
F191 (M.E.)	0·37	0·6	0·62	0·57	0·54	1	1	1	1	1
F192 (M.E.)	0·33	0·4	0·53	0·42	0·42	0·75	1·33	0·87	0·83	0·94

Tests against 4 different batches each of M.M. and M.E. viruses are shown, the titres of the sera being shown in terms of the value obtained for the homologous serum.

Against M.M. the homologous serum is approximately twice as active as either of the two heterologous sera but against M.E. it has less than a third of their activity.

The only other way in which the two viruses can be directly compared is by allantoic inoculation. Here the low power of M.E. to produce infection demonstrable by haemagglutination renders it difficult to get satisfactory results. Nevertheless by using both haemagglutination by allantoic fluid and death of the embryo as indices of infection it was possible to show a distinct advantage on the side of the homologous sera over the heterologous F83.

TABLE 6.

Allantoic neutralization of strain M.E.

Serum.		Serum virus mixtures. S + V.	S/3 + V.	S/10 + V.	S/30 + V.	S/100 + V.	Appropriate neutral mixture.
F83 M.M.	1	0/8	0/8	0/12	3/8	—	S/40 + V
	2	1/3	7/8	8/8	8/8	—	S + V
F191 M.E.	1	0/4	0/4	0/12	0/8	3/8	S/100 + V
	2	—	—	4/8	7/8	8/8	S/20 + V
F192 M.E.	1	0/4	0/4	0/8	0/4	4/4	S/50 + V
	2	—	—	2/4	3/4	4/4	S/10 + V

Three concordant experiments are combined. In the first only haemagglutinin readings at 3 days were made (1). In the second and third both haemagglutinin (1) and specific death of the embryo (2) were recorded. The number of positive findings is shown over the number of embryos used for each mixture.

S = undiluted serum. V = undiluted virus.

The experiments followed the usual form, mixtures of equal volumes of appropriate serum and virus dilutions being inoculated in 0·05 ml. volume into 11 day embryos, 4 being used for each mixture. Tests with M.M. were read at 3 days using fowl cells for test. The M.E. embryos were tested for haemagglutinin against guinea-pig cells at 3 days and at 5 days the embryos were examined for specific lesions and another haemagglutinin test made. Table 6 shows the details of the M.E. test. There were no unusual features of the M.M. tests and only the combined results are shown with those deduced for M.E. from Table 6 in Table 7.

TABLE 7.

Summary of allantoic neutralizations.

	Against M.M.	Against M.E.	
Serum.	1.	1.	2.
F83 M.M.	S + V/5	S/40 + V	S + V
F191 M.E.	S + V/3	S/100 + V	S/20 + V
F192 M.E.	S + V/10	S/50 + V	S/10 + V

Mixtures giving approximate 50 p.c. end points shown. Methods 1 and 2 as in preceding Table.

These results are subject to considerable experimental error but they establish three points with sufficient significance, (1) that all sera prevent infection by M.E. more readily than they do infection by M.M., (2) that the sera are approximately equally active against M.M. and (3) the homologous sera are significantly more active against M.E. especially in preventing death of the embryo, than in F83. It did not appear justifiable to make the extensive experiments necessary to give more accurate quantitative data on these points.

CHARACTER OF EARLIER STAGES IN THE TRANSFORMATION OF "MELBOURNE" to "M.E.".

In order to obtain some idea of the changes in haemagglutinating activity that had taken place in the course of the adaptation of M.E. to the chorioallantois, virus was revived from material which had been dried and stored in cold since 1935 and 1937. The 1935 material was 39th chorio-allantoic passage. At this stage foci on the chorioallantois were noted as definite but no evidence of any lethal effect on the embryo or of haemorrhagic lesions appeared for another 20 passages. Material stored in 1937 was 76th passage virus which had developed the full pathogenicity for the embryo and was essentially the state in which the virus was used in our earlier immunological work.

These dried materials were redissolved in horse serum saline and inoculated amniotically in 11 day embryos. This route was used as the one most likely to allow the regeneration of long-stored material.

The eggs were opened after 4 days incubation at 30° C.

Table 8 shows the agglutinating characteristic of the fluids obtained. The embryos dead from infection with 39th passage virus showed no signs of haemorrhage, and one was tightly bound by the amnion, the other two had 1–3 c.c. of fluid in the amniotic cavity. From one of these embryos its was possible to obtain a little blood from the allantoic vein without contaminating it with extra-embryonic fluids. This blood suspended in saline deposited without any evidence of agglutination.

TABLE 8.

Recovery of earlier passage M.E. strains.

		Fate of embryo.	Allantoic fluids.		Amniotic fluids.		
M.E. 39th passage	27.11.35	†4 †4 †4	320/60	400/200	400/240	400/400	400/200
M.E. 76th passage	3.5.37	†4 †4 S4	0/0	0/0	0/0	0/0	0/0

F/G Haemagglutinating titres are shown.

This recovered strain is therefore hardly distinguishable from M.M. and serological tests have confirmed the resemblance. Tested by haemagglutinating methods against the three ferret sera F83 (M.M.), F191 (M.E.) and F192 (M.E.) the ratio of the antibody titres obtained was 1: 0·72: 0·65 which is close to the values 1: 0·54: 0·42 obtained with M.M.

It has no power to infect embryos by intravenous inoculation.

Of those infected with 76th passage virus two were dead with typical haemorrhagic lesions, the other was alive but showed early haemorrhages. Moderate amounts of lightly turbid amniotic fluid were present but as indicated in the Table it had no power to agglutinate either fowl or guinea-pig erythrocytes. This inactivity was of particular interest in view of the previous failure to obtain agglutination with this strain. Further study, however, indicated that such failure was due mainly to the low titre of virus in the fluids. One of these primary amniotic fluids without agglutinating action had a chorioallantoic titre of 9×10^4. By comparison with other findings with the long passage strain of M.E. this should have shown a guinea-pig titre of about 10.

The failure to do so may have been due to some qualitative difference in the virus or to the inhibitory effect of normal constituents in the amniotic fluid.

Allantoic passage of this strain into 12 eleven day embryos showed when the eggs were opened at 3 days 8/12 fluids capable of agglutinating guinea-pig red cells when undiluted but only one specific death of the embryo. All the positive fluids were of very low titre 0/5, 0/2, 0/1·5 being characteristic F/G titres.

Continued allantoic passage of the strain M.E. (ex 76th passage) gave the representative titres shown in Table 9.

TABLE 9.

Haemagglutinin titres of passage from 76th passage dried material.

Amniotic (ex dried material)				0/0*	0/0	0/0	
1st allantoic passage			89078–83	0/0*	0/2	0/1·5	
2nd	„	„	89312–14	0/8	3/10*	—	
3rd	„	„	89384–87	0/15	0/8	0/8	0/3·5

Subinoculations were made from fluid indicated by asterisk.

The haemagglutinin titres of this substrain have remained too low to make serological study practical. A few experiments have shown that embryos inoculated intravenously are fatally infected with characteristic haemorrhagic lesions at least to a dilution of 1: 10^4 and that the virus is readily neutralized by this route with serum F192.

DISCUSSION.

The strain "Melbourne Egg" possesses a unique combination of characteristics as far as can be judged from the literature. A similarly adapted W.S. substrain produces similar foci on the chorioallantois and the same type of fatal haemorrhagic infection of the embryo. It has, however, retained relatively high virulence for ferret, mouse, and cynomolgus monkey and gives a D type of haemagglutination. M.E. on the other hand is avirulent for ferrets and mice, though still capable of inducing immunizing infections, and gives a type of haemagglutination which is indistinguishable from that of unadapted O strains.

The significance of its resemblance to O type virus in this respect is at present completely obscure; but if our contention (Burnet and Bull, 1943) is correct that the O character is of fundamental importance in relation to the human pathogenicity of influenza virus A, we feel that a close study of M.E. is justifiable even at the present time. The O or pseudo-O character is obviously a secondary change since in the earlier stages of egg passage the strain had the full D character.

An attempt to repeat the transformation to the full M.E. character is at present in progress in the hope that it will be possible to establish whether the change is a progressive one or like the standard O–D change, a sharp discontinuous mutation.

SUMMARY.

The strain "Melbourne egg" of influenza A virus has been re-examined in regard to its power to multiply in various situations in the chick embryo and to the type of haemagglutination produced.

In the early stages of chorioallantoic passage the strain showed typical D haemagglutination: in its fully developed form it gives weak fowl cell agglutination but strong agglutination of guinea-pig or human cells resembling in this respect an unadapted O strain.

The M.E. strain infects embryos to full titre by intravenous inoculation producing characteristic haemorrhagic lesions.

There is a clearly recognizable antigenic difference between M.E. and the substrain of Melbourne adapted to mouse passage.

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ATTEMPTS TO PROLONG THE LIFE OF ENUCLEATED AMOEBAE

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The chemical composition of the surrounding environment greatly influences the vital processes taking place in a living cell. Mast (1931) has found that very dilute salt solutions, both single and mixed, prolong the life of amoebae kept without food, as compared with the length of life in pure water. The experiments described in this paper were aimed at prolonging the life of enucleated amoebae.[1] To this end, the amoebae were both immersed in and injected with solutions of the various substances tested. The literature on the subject is small, the one paper of Lynch (1919). He found that 1 p.c. solutions of glucose and laevulose prolonged the life of both nucleated and enucleated amoebae in the absence of food. (As enuculeated amoebae are unable to digest food, Lynch (1919), Clark (1943), it is necessary to compare nucleated and enucleated fragments under starvation conditions.)

Lynch also found that 0·1 p.c. urea added to the glucose was still more beneficial to the nucleated amoebae, although it was toxic to the enucleated. Ammonium nitrate (0·01 p.c.) was found to be without effect. As some of the nucleated amoebae underwent fission in the glucose-urea solution, Lynch concluded that the medium supplied not only carbohydrate for energy but also nitrogen for the synthesis of new protoplasm. He clouds the issue by stating that although fission was observed in this solution, there was never any growth. The occurrence of fission is no proof of the utilization of nitrogen contained in the medium, for Mast (1931) has observed it to take place in the following solutions: pure water; K_2SO_4 N/1000; Na_2SO_4 N/1000; NaCl N/1000; NaOH + NaH_2PO_4, pH 4·5 and others. As most organisms prefer to excrete urea rather than to utilize it, it is difficult to believe that Lynch's amoebae were actually using the urea nitrogen for purposes of synthesis. However, on the grounds that urea appeared to be utilizable to nucleated, but not to enucleated amoebae, Lynch concluded that an enucleated amoeba is unable to synthesize.

Culture methods and ways of obtaining enucleated amoebae have been outlined in earlier communications (Clark, 1942, 1943). Before enucleation, the amoebae were kept in filtered tap water for 48 hours, or longer if necessary, until all food vacuoles were eliminated. The water used for making up the various solutions was distilled from a copper still and thence twice redistilled from an all-Pyrex glass still. Chemicals used were A.R. Standard (British Drug Houses Ltd.). All the experiments were carried out at 15°–18° C. A control experiment revealed that the enucleated amoebae would live for an average of 7 days in tap water.

SINGLE AND MIXED SALT SOLUTIONS.

The effects of salts on normal amoebae have been investigated by Chambers and Reznikoff (1926), Reznikoff and Chambers (1927), and Mast (1928, 1929, 1931). In the present experiments, enucleated and control nucleated amoebae were immersed in and injected with salts ranging in concentration from M/10 to M/1280.

Potassium and sodium chlorides. Immersion of enucleated amoebae in solutions more concentrated than M/80 results in a rapid withdrawal of any pseudopods present, followed by round-

[1] In this paper, the letters N and E are frequently used as abbreviations for nucleated and enucleated amoebae respectively.

ing up and inactivity. Cytoplasmic viscosity is reduced and clumping of the crystals occurs. No streaming at all can be observed and disintegration occurs, rapidly in the potassium solutions, more slowly in the sodium. In M/320 KCl, the amoebae may show very slow, irregular streaming and temporary attachment to the substrate but disintegration occurs within 30 hours. In M/320 NaCl, activity and degree of attachment are both increased slightly and the amoebae live for 5 days. During this period, no clumping of crystals is observed. In M/1280 KCl, length of life is about 4 days and activity is equivalent to that found in enucleated amoebae maintained in tap water. Similarly, as the dilution of NaCl increases, behaviour and length of life approximate to those of control amoebae. The injection of KCl and NaCl solutions produce effects similar to those obtained by Chambers and Reznikoff with normal amoebae. The injection of small amounts of dilute saline was found to have no effect on the length of life.

Calcium and magnesium chlorides. In M/10 $CaCl_2$, the enucleate amoebae become completely inactive, if they are not already so, and there is a marked increase in the viscosity of the plasmagel (estimated by the degree of Brownian movement). $MgCl_2$ produces a similar effect. In both solutions death occurs within 30 hours. In M/40 $CaCl_2$, activity may be increased considerably for 15 to 30 minutes, but quiescence eventually sets in. A solution of $MgCl_2$ of similar concentration is less toxic than the calcium solution but does not exert a stimulating effect on activity. However, as the dilution increases, the calcium salt becomes the less toxic and has a favourable effect, not only on the activity, but also on the length of life. In M/500 $CaCl_2$, 5 enucleated amoebae lived for an average of 8·7 days. In the same solution, 5 nucleated, control amoebae lived for 10·2 days. The solutions of $MgCl_2$ of similar strength were never so effective. The injection of $CaCl_2$ and $MgCl_2$ solutions produced similar effects to those observed in normal amoebae by Chambers and Reznikoff—gelation and subsequent pinching off of the injected region. Recovery followed the injection of $CaCl_2$ but $MgCl_2$ was always toxic. The injection of a small amount of M/1280 $CaCl_2$ was without effect on the length of life of the amoebae.

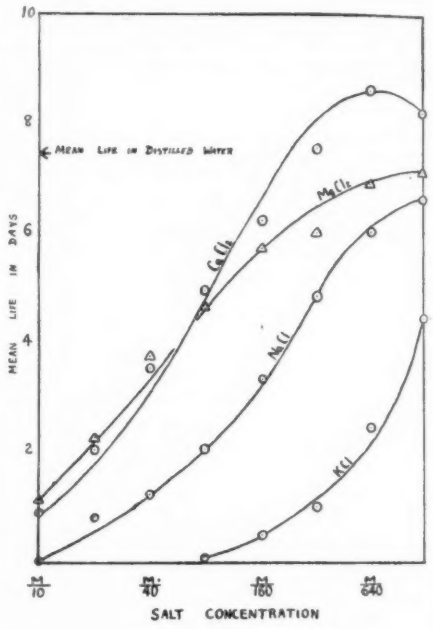

Fig. 1.

The most favourable solution, both from the viewpoints of stimulating activity and of increasing the length of life is M/500 $CaCl_2$. No solution was found to be very effective in strengthening the attachment of the amoebae to the substrate.

Although this calcium solution appears to be favourable, it must be borne in mind, however, that its stimulating effect on activity is only slight. Even in M/500 $CaCl_2$, an enucleated amoeba is still a relatively inactive cell and, on infrequent observation, is generally found to be quite motionless and spherical.

In Fig. 1 the relation between saline concentration and the mean length of life of an enucleated amoeba is indicated by means of a graph. Each point represents the mean life period of 5 enucleated amoebae paced in the particular saline solution immediately after enucleation. The curves are substantially similar to those given by Chambers and Reznikoff (1926) for normal, nucleated amoebae. The only significant difference is that a dilute solution of calcium chloride appears to be far more favourable to enucleated than to nucleated amoebae. If it should prove possible to demonstrate that the nucleus of an amoeba contains relatively large amounts of calcium, by microincineration for example, this observation might become highly significant.

Mast (1931) has found that a balanced solution of mixed salts is more favourable to normal amoebae than a single saline is. Repeating his experiment, but using enucleated amoebae, balanced solutions were prepared containing sodium hydroxide, primary sodium phosphate,

calcium chloride and potassium chloride. The results are given in Table 1, the figures again representing the mean life span of five enucleated amoebae placed in each of the solutions. Control figures are for the five nucleated sister halves.

TABLE 1.

Balanced solutions of NaOH, NaH_2PO_4, $CaCl_2$ and KCl pH 6·8.

Concentration of Na.	Concentration of Ca and K each.	Mean life in days. N.	E.
N/100	N/2,000	7	5
N/500	N/10,000	9	7½
N/1,000	N/20,000	10½	8
N/5,000	N/100,000	8	5½
Control (distilled water)		8	5

The most favourable solution was found to be one in which the sodium ion concentration was N/1000 and the calcium and potassium ions each N/20000. In no solution, however, did the relative increase in life span of E exceed the similar increase in N. The nucleated amoebae still outlived the enucleated by about two or three days.

GLUCOSE, UREA AND PEPTONE.

The effects of glucose, urea and peptone on the length of life of E are given in Table 2. To prevent the development of a large bacterial population, the amoebae were placed in fresh quantities of the respective solutions every 12 hours. As in the case of the saline solutions, the life period is the mean observed in 5 amoebae.

TABLE 2.

Solution.	Mean life in days. N.	E.
1 p.c. glucose	8	6½
0·1 p.c. glucose	7½	6
0·1 p.c. urea	5½	5
0·01 p.c. urea	7	6
1 p.c. glucose + 0·1 p.c. urea	7½	5
0·1 p.c. glucose + 0·01 p.c. urea	8	5½
1 p.c. peptone	5	4½
0·1 p.c. peptone	6	5
1 p.c. glucose + 0·1 p.c. peptone	6½	5
Distilled water	7½	6

The glucose solutions were found to be beneficial to a slight extent to both E and N but the effect was not as great as that obtained by Lynch. Experiments are now being carried out to determine whether the addition of glucose to the medium has any effect on the rate of oxygen consumption of the amoebae.

All the solutions containing urea appeared to be toxic, which is not in harmony with Lynch's view that nucleated amoebae can utilize urea in small amounts and that its addition to the culture medium is beneficial. As the injection of the peptone solutions was usually followed by fatal results, its slight toxicity when present in the external medium was expected. Glucose was not toxic after microinjection, but such an injection never prolonged the life of either nucleated or enucleated amoebae more than that obtained by immersion. Microinjection of urea was usually followed by the death of the amoeba within a few hours.

Two other attempts to prolong the enucleated amoeba's life may be briefly mentioned. Immersion, and injection of amoebae with a dilute solution of thymonucleic acid (prepared in this laboratory) had no effect on the viability of the amoebae. This experiment is comparable with that of Harvey (1934), who found that the injection of nucleic acid, adenine and guanine, into *Arbacia* eggs failed to induce parthenogenetic merogones to develop beyond the blastula stage.

In the second experiment, enucleated amoebae were injected with and immersed in an extract obtained from nucleated amoebae in the following manner. Several thousand amoebae were centrifuged and placed in a drop of water in a small watch glass. Very fine emery powder was added and the amoebae were ground up with a glass rod. The process was carried out under a binocular microscope, as by observation it was possible to be sure that the amoebae were really disintegrated. The final extract was allowed to stand for one hour, and was then centrifuged.

Both immersion and injection greatly increased the activity of the enucleated amoebae for one to two hours, but the extract was toxic, for the amoebae only lived for an average of 3½ days. The toxicity of the mixed cytoplasmic-nuclear extract is not altogether surprising as Chambers (1922) found that nuclear fluid from echinoderm eggs, when injected into intact eggs, produces rapid cytolysis.

I regard it as highly significant that in none of the experiments did the enucleated amoebae survive as long as the nucleated. Like Lynch, I have found that size is an important factor in determining how long an enucleated amoeba will survive. The larger the fragment, the longer the survival period. For this reason, an amoeba enucleated by the direct removal of the nucleus micrurgically lives longer than one enucleated by bisection of a normal individual into nucleated and enucleated fragments. In association with the longer life, there is also greater activity in large enucleated amoebae than in small ones. In the experiments reported in this paper, all the enucleated amoebae were obtained by bisection, the greatest care being taken to ensure that the resulting E and N were of similar size. Hence the differences in viability shown by E and N in the different solutions cannot be ascribed to size differences. This being the case, the experiments do not confirm Lynch's conclusion that an enucleated amoeba lives as long as an amoeba deprived of food. In other words the ultimate death of an enucleated amoeba is not due merely to its inability to digest and assimilate food. That inability to digest food is not the prime cause of death was also demonstrated experimentally in the following manner.

A large amoeba was kept for 3 days in distilled water until all food vacuoles were eliminated. The nucleus was then removed by a micro-pipette and the amoeba returned to distilled water. Two days later the enucleated amoeba, together with a normal one, was placed in tap water containing a thick suspension of *Chilomonas*. After 4 hours, E had two food vacuoles containing several Chilomonads in active movement. Seven hours later, the Chilomonads were motionless but showed no other signs of being digested. Using a very fine pipette, a minute droplet of fluid was aspirated from a food vacuole of the nucleated amoeba, in which the Chilomonads were partially digested, and injected into one of the food vacuoles of the enucleated amoeba. One hour later the Chilomonads in the injected vacuole had fragmented, whereas those in the second vacuole were unchanged. On the following day the Chilomonads in the one vacuole were completely digested except for a few droplets, whereas those in the other vacuole were still intact. The amoeba died 4½ days after enucleation. This experiment was attempted on ten occasions. Seven times it failed for technical reasons, namely the rupturing of the food vacuole on the introduction of the pipette. In the remaining three cases, the amoebae lived for 4½, 4 and 5 days respectively. Since the average life of an untreated E in tap water is seven days, it follows that supplying digestive enzymes to an enucleated amoeba fails to prolong the life. In fact, in the three successful experiments, the enzymes seemed actually to decrease the length of life by about two days. The experiment, of course, does not tell us anything of the ability of E to utilize digested food for the purposes of synthesis.

In the saline immersion experiments, the amoebae had no food vacuoles and there was no animate food present in the medium. Consequently, any reserves of substances utilizable as sources of energy would be used for maintaining the biological equilibria of the amoeba, such as any energy that might be required to maintain membrane semipermeability. It is not to be expected, therefore, that under these starvation conditions energy would be used for synthesizing new protoplasm. As the mean life of a starved E is less than that of a starved N, it follows that an inability to synthesize is not necessarily the cause of the ultimate death of E any more than is the absence of digestive enzymes. A very active E may ingest and partially digest food but no growth (increase in volume) is ever observed. The lack of growth may well be due to an inability on the part of E to synthesize new protoplasm, but it must be emphasized that this inability to synthesize is not the direct cause of the ultimate death of the amoeba, except in so far as cellular differentiation may have its ultimate basis in syntheses.

As an alternative to Lynch's suggestion that an inability to synthesize is the cause of death, the following theory is offered. The removal of the nucleus means

the removal of genes which may be responsible, not only for the appearance of differentiation but for its maintenance. In their absence, the ultramicroscopic differentiation of the cytoplasm regresses and biochemically active surfaces are no longer maintained, with the result that the amoeba is unable to utilize what food reserves it may have and, eventually, is unable to carry on the fundamental processes required to keep the cell in the living condition.

SUMMARY.

Several saline solutions, single and mixed, have been tested in order to find one capable of prolonging the life of an enucleated amoeba.

M/500 $CaCl_2$ is the most effective saline solution, enucleated amoeba living in it for an average of $8\frac{1}{2}$ days.

Glucose is slightly beneficial to enucleated amoebae. Peptone and urea are toxic.

The ultimate death of an enucleated amoeba is not due to an inability to digest and assimilate food.

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THE RESPONSES OF ENUCLEATED AMOEBAE TO STIMULI

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In two recent papers (Clark, 1942, 1943) an account was presented of the general characteristics of enucleated amoebae. The present communication deals in a more detailed manner with the behaviour of enucleated amoebae and their responses to external stimuli. Verworn (1890), Balbiani (1888) and Jennings and Jamieson (1904) found that the reactions of enucleate ciliate fragments to chemicals, heat and electricity are normal, while recently Tartar and Chen (1941) have reported that the complicated mating reactions of enucleate fragments of *Paramoecium bursaria* are the same as those of intact individuals. Earlier investigators of the effects of enucleation on the behaviour of amoebae have, in general, confined their activities to a consideration of one type of stimulus alone. Hence their not always harmonious conclusions on the normality or abnormality of the responses can be valid only for that one type of stimulus. Gruber (1886) and Hofer (1890) found that the locomotion of enucleated amoebae was abnormal, but were silent on the subject of irritability. Stole (1910) showed to his own satisfaction that enucleated amoebae were as irritable as nucleated ones and that their responses and locomotion were normal. The response he considered was the development of the stellate condition (Reizzustand), which is obtained when, as a result of gentle agitation, an amoeba loses its attachment to the substrate. Willis (1916) asserts that an enucleated amoeba shows no orientation in a beam of light and he believes this may be the result of abnormal locomotion and lack of attachment to the substrate. Becker (1926) was unable to confirm Stole's findings on the production of the stellate condition. He believes that enucleated amoebae, although irritable, do not give a normal response, probably because of insufficient protoplasmic turgidity.

Enucleated amoebae were obtained by bisecting normal individuals by means of a glass thread. Thus, for each enucleate amoeba there is a corresponding nucleated sister half, whose responses act as controls. For detailed information on the behaviour of normal, nucleated amoebae, reference should be made to the papers of Jennings (1904); Schwitalla (1924); Mast (1931 a); Mast (1931 b); Mast and Prosser (1932) and Hahnert (1932).

THE RESPONSE TO STIMULATION WITH A NEEDLE.

To examine this response the amoebae were stimulated with a fine glass needle controlled by means of the micromanipulator. The normal response of a nucleated amoeba is an avoiding or negative one. The amoeba changes its course and moves away in another direction, the angle the new course makes with the old one depending upon the strength of the stimulus. If it is severe and persistent, the amoeba moves directly away from the point of stimulation. Typical examples of the responses obtained from enucleated amoebae will be given.

An amoeba was cut in halves and both fragments were placed together in a watch glass so that all the reactions obtained from the enucleated portion could be compared at once with those shown by the nucleated, sister half. An hour and a half after enucleation, E[1] was lightly attached

[1] The letters N and E are frequently used as abbreviations for nucleated and enucleated amoebae respectively.

and was showing slow streaming. While being observed under a magnification of 350 (water immersion lens) the tip of the pseudopod was touched with the needle. The pressure was sufficient to depress the plasmalemma of the hyaline cap, pushing it back against the plasmagel sheet. Streaming in the anterior end ceased in 5 seconds and reversed after an additional 6 seconds. It was slow however, and no new pseudopod was formed for 45 seconds. A similar stimulus applied to the nucleated half produced a cessation of streaming in 3·5 seconds, reversal in 7 seconds and new pseudopod formation in 12 seconds. In both cases the new pseudopod formed was at an angle of about 45 degrees with the original direction of streaming. On repeating the stimulus but using less force, the plasmalemma being only slightly depressed, the enucleated part ceased streaming in 4 seconds but this was resumed in the original direction 5 seconds later. The nucleated half ceased streaming in 2 seconds, resuming it again in the same direction 4 seconds later.

In other experiments, the amoebae were stimulated after they had been enucleate for several hours. The following account is for an amoeba bisected twenty-six hours previously. Before the experiment the enucleated portion was elongated but was unattached and inactive. Gentle stimulation produced streaming away from the needle lasting for four minutes. Eight minutes later, streaming was resumed in the same direction but as there was no attachment, progressive locomotion did not occur. A few minutes later the amoeba became inactive. After an interval of twenty minutes, a gentle stimulus was again applied, resulting in streaming away from the needle lasting for less than a minute. A very strong stimulus was then applied, the needle actually rupturing the plasmalemma. Immediate and rapid streaming away from the needle followed and lasted for three minutes. As before, the amoeba showed no signs of attachment and was thus unsuccessful in moving far away from the needle. The reactions of the nucleated half were found to be normal. From this experiment it is seen that streaming in an inactive, enucleated amoeba can, at certain times, be induced by stimulation. As a result of 35 experiments, it was found that this effect is not observed in amoebae that have been spherical, unattached and inactive for some time. Such amoebae show no response at all to a mechanical stimulus, regardless of its severity.

The positive reaction sometimes shown by an amoeba when it comes into very light contact with a solid body is difficult to observe even in the normal amoeba. Nevertheless some evidence is available to show that a positive response will sometimes be given by the enucleated amoeba. If the amoeba is mounted in a medium containing solid objects such as debris, threads of algae or cotton, provided it happens to be active at the time it will stream in contact with these objects. On one occasion a particularly active enucleated amoeba was at hand and it was mounted together with a small ball of *Spirogyra* threads. The amoeba soon attached itself to the surface of the ball and streamed around and over it for more than half an hour. This display was so convincing that when the amoeba finally became inactive, it was fixed and stained in order to verify the absence of the nucleus. Unfortunately the majority of enucleated amoebae show neither the activity nor the power of attachment to a sufficient degree to yield this type of response, but the fact that some individuals do become active enough to show the response indicates that enucleated plasma is capable of responding in a normal manner, and that whether it does so or not depends upon the activity rather than upon any inherent inability to show the response.

THE PRODUCTION OF THE "REIZZUSTAND" OR STELLATE CONDITION, A RESPONSE TO AGITATION AND LOSS OF ATTACHMENT.

This is the response considered by Stolc (1910) and Becker (1926). If an active and firmly attached amoeba is sucked up into a pipette and expelled again, it first of all withdraws all its pseudopods and contracts. This condition lasts for a minute at the most. Soon the amoeba sends out long, thin pseudopodia in all directions, resulting in a stellate or radiate condition which Stolc refers to as the Reizzustand, the stimulated condition. The extension of the pseudopodia seems to be an adaptive response to loss of attachment, for these long, thin processes greatly increase the chances of the amoeba coming in contact with some solid surface. Mast (1928) found that in distilled water an amoeba may remain in this condition for more than twelve hours, during which period little or no streaming can be observed.

Stolc's contention that the enucleate amoeba gives a normal response is only partly correct. Like the response to contact with a needle, the stellate condition can be produced only under certain conditions. The conditions are that the amoeba must be firmly attached and active before stimulation. The following account of some typical cases illustrates the features of the response. The number of each

experiment refers to the cultures from which the amoebae were taken and the day and month of enucleation.

Twenty-four hours after bisection the nucleated half of 10·27/5 was firmly attached and streaming rapidly in proteus fashion. E was lightly attached but the streaming, of limax type, was very slow. Both were transferred by pipette to a watch glass of distilled water. Within three minutes, N became stellate, extending four long, thin pseudopods. E was inactive for five minutes, but then extended three short, blunt processes. This was regarded as a response approximating to normality.

On the third day after bisection, N was again firmly attached and active. E was neither active nor attached. After transference to a second watch glass, N gave an excellent response, whereas E gave no response at all.

Half an hour later, N was still stellate whereas E commenced streaming in limax fashion but was unattached.

Number 10·6/6 was used four days after bisection. Both N and E were active but only N was attached, and gave a good reaction, whereas E extended only one thick pseudopod.

Number 8·6/6 was examined thirty-six hours after bisection. N was active and attached. E was lightly attached but was not streaming. A typical response was obtained from N, whereas E extended two short pseudopods only after 8 minutes.

These three examples were selected from a series of twenty-five experiments, for they illustrate the four possible states in which the amoeba may be before it is stimulated. It may be active and attached, active but unattached, attached but inactive or both inactive and unattached. Only if the enucleated amoeba is in the first condition, i.e. active and attached, will it give an approximately normal response to agitation and loss of attachment (e.g. 10·27/5).

A better response is obtained from attached inactive amoebae than from active unattached ones, while no response at all is obtained from completely inactive and unattached individuals.

The length of time elapsing since enucleation was found to have no direct effect on the ability of the amoeba to respond to the stimulus. In six of the twenty experiments referred to, responses, admittedly of a poor nature, were obtained from amoebae enucleated for more than seven days. With lapse of time, however, the enucleated amoebae generally tend to become less active and consequently less responsive to a stimulus.

The character of the pseudopod extended by an enucleated amoeba showing a response is probably significant. Instead of long, thin processes, typical of nucleated amoebae, they are always broad and usually short. I can offer no satisfactory explanation for this feature.

THE RESPONSES TO CHEMICAL STIMULATION.

Amoeba is very sensitive to changes in the composition of the medium in which it is moving, and always shows a negative response when it comes into a region in which the chemical composition differs from that to which it is accustomed. Various explanations for the mechanics of the response have been suggested, most of which are based upon surface tension considerations.

For stimulation, the amoebae were placed in a watch glass of culture water and the solution of the chemical was applied by means of a fine pipette attached to the micromanipulator. With this arrangement the stimulus was highly localized. The chemicals tested were 1 p.c. solutions of methyl green, copper sulphate, sodium hydroxide and hydrochloric acid.

The enucleated amoeba shows a negative reaction to a chemical stimulus, provided that it is streaming at the time the stimulus is applied. In all cases when the amoeba was streaming, reversal was obtained within 10 seconds. In a few cases involving particularly active amoebae, it was possible to follow stimulation at one end, which resulted in reversal of direction of streaming, by stimulation at the other end, resulting in a second reversal, the amoeba then streaming in the original direction. For this type of experiment the monopodal or limax form characteristic of an enucleated amoeba is very suitable.

More than two successive stimuli cannot usually be applied successfully to the same enucleated individual. Even a comparatively weak stimulus, while producing a normal negative response, frequently results in the amoeba passing into an inactive condition. Such a condition can be produced in a nucleated amoeba only by severe and repeated stimuli. Thus, either the enucleated amoeba is very much more sensitive to the deleterious effects of chemicals than is the nucleated amoeba, or owing to some metabolic disturbance, it is unable to stream for any length of time. Furthermore, the enucleated amoeba does not give a response quite so readily as a normal individual. Cessation or reversal of streaming is always slower in appearing in the enucleated amoeba than it is in the nucleated, sister half. Whereas a nucleated amoeba will cease streaming almost immediately it comes in contact with the chemical, an enucleated amoeba will often persist in streaming towards the chemical for as long as 10 seconds.

THE RESPONSES TO LIGHT.

If a normal amoeba is subjected to a rapid increase in illumination, streaming ceases. Mast (1931a) calls this the shock reaction. On the other hand, exposure to a horizontal beam of light of constant intensity causes the amoeba to orient away from the source. Mast believes the two types of response are fundamentally the same, as the stimuli in both cases inhibit the formation of pseudopods in the illuminated area by increasing the amount of plasmagel and the elastic strength of the plasmagel tube. Rapid increase in the intensity of illumination is followed by inactivity, while a gradual increase results in a decreased rate of locomotion. If a small increase in illumination is repeated several times, the effects are cumulative. Illumination of the hyaline cap produces no effect but a reaction is obtained if any other part of the amoeba is illuminated. In this work, only illumination of the plasmagel sheet immediately behind the hyaline cap was considered.

The source of light for the localized stimulus was a 250-Watt gas-filled lamp. The whole of the glass surface was blackened except for a small circular area directly opposite the filament. The light from the lamp was reflected through the substage condenser of the microscope by means of the plane mirror, the aperture of the diaphragm was reduced, and a small section of the image of the lamp-filament focussed on to the amoeba. By rotating the mirror the image of the filament could be moved to any part of the field. The amoebae were mounted under vaselined cover-slips in the usual way and the observations were made under a magnification of 420. In all experiments the intensity of the source of light was maintained constant.

Illumination of the plasmagel sheet in an active enucleated amoeba results in cessation of streaming. In the following accounts it is to be understood that as soon as this occurred the light was turned off. Thus the figures given for the reaction times indicate the length of time the plasmagel sheet was illuminated before streaming ceased.

Amoeba number 3·1/4 was used one hour after bisection. Both E and N were active but only N was attached. Stimulation of E caused cessation of streaming in 6·5 seconds. The streaming was resumed in the original direction 62 seconds after removing the stimulus. A second stimulus resulted in cessation of streaming in 12 seconds and activity was not resumed for seven minutes. Stimulation of N caused streaming to cease in 5 seconds but it was resumed 10 seconds later. In the meantime E had recommenced streaming and a stimulus caused cessation in 10 seconds followed by almost immediate reversal. The reverse stream lasted for 15 seconds, when movement was resumed in the original direction.

Two hours later an attempt was made to detect the accumulative action of a stimulus. As soon as the amoeba had resumed streaming after one stimulation, it was immediately restimulated. The successive reaction times obtained were:

Nucleated part: 8·0″, 6·2″, 7·9″, 4·3″. *Enucleated part:* 6·5″, 12·5″, 10·8″, 12·0″.

An indication of an accumulative effect can be seen in the figures for the nucleated half but not in those for the enucleated half. This may be related to the fact that between stimuli N resumed streaming almost at once, whereas E frequently became inactive for as long as 2 minutes before streaming recommenced. Amoeba number 3·2/4, used 3 days after bisection, was stimulated in a similar manner, but the plasmagel sheet was continually illuminated until not only cessation of streaming, but complete reversal, had been produced. As soon as the plasmasol commenced to flow away from the light, the stimulus was removed. Under these conditions, the reversed streaming lasted for a short while only and was followed by streaming in the original direction. The reaction times for several successive trials were:

Nucleated half: Cessation of streaming: 3″, 3″, 2″, 2″. Commencement of reverse streaming: 8″, 6″, 6″, 3″.

After the fourth time, a new pseudopod was extended at right angles to the original direction.

Enucleated half: Cessation of streaming: 9″, 5″, 5″. Commencement of reversed streaming: 15″, 10″, 8″.

After the third stimulus streaming was not resumed. As E was still inactive half an hour later, a portion of the plasmagel at the anterior end was illuminated. Streaming away from the light occurred after a latent period of 20 seconds, and lasted for several minutes. During this period of activity induced by the stimulus, it proved possible to stop the streaming temporarily by further stimulation. The reaction times for three successive tests were 7·5″, 8·8″ and 7·0″. A fourth test was made to see if continued illumination would result in a reversal of streaming. This occurred in 12·6″, and the direction of reversed streaming was maintained for rather less than a minute, when the amoeba again became inactive. After a further interval of ten minutes the amoeba was stimulated at the end previously posterior. Streaming away from the light occurred in 7″ and lasted for a quarter of a minute. All further attempts to induce activity failed.

The above two examples contain all the features of the responses to sudden change in illumination that were obtained from enucleated amoebae. These responses were obtained many times in thirty different amoebae, and so can be regarded as being typical. The responses of an active enucleated amoeba are depressed or slower, but otherwise normal, although in no case has a very well-defined accumulative effect of stimuli been observed. Oft-repeated stimuli result in the amoeba passing into an inactive condition and, as we have already seen, this occurs frequently in experiments on chemical stimulation.

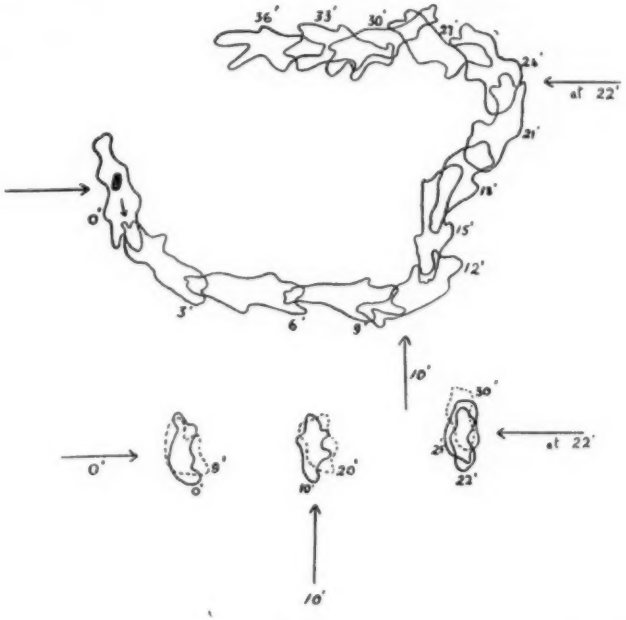

Fig. 1.

The second type of response to light is that obtained when an amoeba is exposed to a horizontal beam of constant intensity. The experiments required the use of a suitable container in which the amoebae could be exposed readily to the beam of light. A small observation chamber was made by cementing on to a slide four equal strips of glass, 20 mm. × 4 mm. The floor of the chamber and three of the glass strips were given several coats of Indian ink. The remaining side was coated with Indian ink except for a small oblong slit 2 mm. × 10 mm., this slit serving to allow a beam of light to pass into the chamber. A lid of stout cardboard was cut to the required shape and was then coated with Indian ink.

When making the observations, the amoeba was placed on a cover-slip that had small legs of wax at each corner. The cover-slip could then be turned around so that the amoeba was exposed to light from four different directions alternately. A lens and mirror system served to reflect a beam of sunlight into the chamber. Any orientation shown by the amoeba was observed through a small hole in the lid of the chamber, the hole being just large enough to permit the focussing of a Leitz 3 objective. As the edge of the objective fitted snugly into the hole, no stray light entered the chamber from this quarter. To prevent the amoeba from becoming too warm when exposed to the sun's rays, two glass tanks filled with a dilute solution of copper sulphate were placed between the mirror and the lens.

The manner in which the nucleated amoeba orients when under such conditions is shown in Fig. 1. The outline of the amoeba was drawn at 3 minute intervals with the aid of a camera

lucida. After each ten minute interval the cover-slip was turned through ninety degrees, the direction of the beam of light being shown by arrows in the figure. The orientation is quite definite. On the other hand, the sister, enucleated half showed no signs of orientation. Indeed, the activity was so slight that it was not possible to reproduce the outline of the amoeba more frequently than every ten minutes. Even so, there is a large amount of overlapping of the figures.

In nearly all of my experiments the enucleated amoebae showed absolutely no signs of orientation whatever. On two occasions however, they did make a few movements away from the light but, as streaming was always of short duration and attachment either weak or absent, it was not possible to detect a well-defined orientation. Nevertheless the response was an avoiding one and, in that respect at least, was normal.

It must be concluded that an enucleated amoeba does not orient in a beam of light, a conclusion in agreement with that of Willis (1916), but it is probable that the failure to show any orientation is due to the absence or irregularity of locomotion and to the absence of firm attachment of the amoeba to the substrate.

THE RESPONSES TO HEAT.

There are two ways in which an amoeba can be subjected to heat. Either the temperature is raised suddenly in a localized manner by bringing a red hot needle close to the amoeba, or the temperature of the amoeba's entire environment is gradually increased by means of a warm stage. Schwitalla (1924) and Mast and Prosser (1932) have shown that the rate of locomotion in a normal, nucleated amoeba increases with rising temperature up to a certain point, after which it falls to zero. Mast and Prosser also state that the locomotion-rate/temperature curve shows a maximum at 24° C., followed by a minimum at 28° C. and a second maximum at 30° C. At 32°–33° C. there is no locomotion. This cessation of locomotion is preceded by the gradual withdrawal of all pseudopodia and an increase in the ratio of plasmasol to plasmagel. At 34° C. there is an entire and irreversible coagulation.

A Hellige electrically-heated stage was used. Temperature differences between the stage and the slide were reduced to as low a value as possible by placing a film of oil between them, thus replacing the air space normally present. In the experiments to be described, the amoebae were first used twenty-four hours after enucleation, unless otherwise stated. Owing to the fact that the enucleated amoebae were rarely attached, it did not prove possible to measure the rate of locomotion with the accuracy to be found in the paper of Mast and Prosser, but in order to obtain some measure of the degree of activity, the rate of extension of the anterior pseudopod was measured in both nucleated and enucleated individuals. The rate of increase in temperature was maintained as constant as possible as all experiments.

The enucleated amoebae showed a gradual increase in activity up to about 27° C., after which pseudopodia were gradually withdrawn and streaming ceased. In a few cases, a small amount of streaming was observed in the internal protoplasm at temperatures as high as 31°–33° C. but was only of a few seconds duration and, not being accompanied by the extension of a pseudopod, could not be regarded as locomotion. In five experiments, the amoebae were at times fairly well attached and showed over a period of several minutes a rate of locomotion comparable with that of nucleated individuals. In the remaining fifteen experiments, the enucleated amoebae showed only fleeting attachment. In Table 1 are given the figures obtained in 20 experiments.

In the first five experiments, both E and N were attached and hence the figures represent the mean rate of locomotion at that particular temperature. In the remaining fifteen experiments, only N was attached and, in order to make a valid comparison between the two types of amoebae, it was necessary to measure the rate of extension of the most anterior pseudopod. The true rate of locomotion in an attached amoeba is rather less than the rate of extension of the anterior or leading pseudopod.

In all cases, the enucleated amoebae moved or extended pseudopods rather more slowly than the nucleated ones, but the most interesting point is that on no occasion did the increase in temperature fail to increase the activity of the enucleated amoebae. Furthermore, enucleated amoebae that were inactive at root temperature always became active, if only for a few minutes, at the higher temperatures. The frequency of rupture of the plasmagel sheet (i.e. the rate at which the stream of plasmasol breaks through the plasmagel at the anterior end and enters the hyaline cap), which Mast and Prosser (1934), using normal amoebae, found to depend on temperature as well as on salt and hydrogen ion concentration, always increased in both E and N as the temperature increased. However, the ruptures were invariably of a much more violent character

in E. When the temperature exceeded 25° C., each rupture was often so severe as to be followed by a sudden, violent gush of plasmagel through the opening. Such violent ruptures were rarely encountered in nucleated amoebae and only when the temperature reached 29° C. It was also found that the E half generally became inactive at a lower temperature than the N, especially if it had already survived several days since enucleation.

TABLE 1.

Time in minutes.	Temperature.	Mean rate of locomotion. (Microns per second)		Mean rate of extension of pseudopod. (Microns per second)	
		N.	E.	N.	E.
0	16	2	—	2·2	—
10	18	3	0·5	2·8	0·8
20	20	3·3	1	3·1	2·3
30	22	3·8	2·3	3·8	3·4
40	24	4·3	3·5	4·4	3·9
50	26	4·8	3·8	4·8	4·2
60	28	2·3	1·8	2·7	2·7
70	30	1·5	0·5	1·8	1·0
80	32	—	—	—	—
		Mean of five experiments.		Mean of fifteen experiments.	

If a high temperature sufficient to cause coagulation was not maintained longer than a few minutes (e.g. three minutes at 33° C.) both nucleated and enucleated halves showed complete recovery. A period of about two hours was required to elapse before they presented a normal appearance. Without exception, the nucleated halves recovered more rapidly and at a higher temperature than the enucleated ones. In one experiment, in which E and N had been heated to 34° C. and maintained at that temperature for 2 minutes, N recommenced to stream at 25° C., whereas E was inactive until 22° C. had been reached. The stage was 20 minutes cooling from 30·2°–25·0° C., and a further 15 minutes cooling to 22° C. Both the amoebae concerned were alive and active 24 hours later.

In about 20 p.c. of the experiments, the enucleated half on cooling to about 27°–26° C. showed partial cytolysis, the plasmalemma rupturing and allowing some of the cytoplasm to flow out. In two cases out of twenty, the cytolysis was complete, the amoebae disintegrating before any repair of the plasmalemma could occur.

In examining the response of the amoeba to a sudden rise in temperature, a small needle was heated in a Bunsen flame until it glowed. It was then touched lightly against the coverslip of the preparation a short distance away from the amoeba, which was observed continually during the process.

If the needle is not too close to the amoeba, the reaction consists of a marked contraction of the plasmagel on that side of the amoeba nearest the needle. This is followed by rapid streaming away from the needle. The response is thus negative, and is similar to that obtained from the amoeba when it is stimulated by a microneedle. The reaction of an active, enucleated amoeba is quite normal. If the wire is very close, the initial streaming may be towards the wire, followed by rapid reversal. A similar response to severe stimulation was obtained from a nucleated amoeba. (See also Jennings, 1904).

If the enucleated amoeba is inactive and unattached at the time the stimulus is applied, a sudden, violent streaming of plasmasol occurs at first towards and then away from the needle. Once this initial stream has occurred, no further activity takes place and the amoeba makes no more attempts to move away from the wire. The initial burst of activity is probably due to the severe contraction of the plasmagel. As in the case of other stimuli, the response of the enucleated amoeba to a sudden rise in temperature is correlated with the ability of the amoeba to show continuous streaming. If at the time of stimulation the amoeba is active, the response it shows is the same as that of a normal individual.

THE RESPONSE TO ELECTRICITY.

When stimulated with a weak electric current an amoeba will show a definite "make" reaction. On the other hand, no reaction at break can be observed. Before the make-reaction is obtained the current must be of a certain minimum strength and must flow for a certain time, the reaction time. For a nerve-muscle preparation this is inversely proportional to the strength of the current and is a

case of the law formulated for striated muscle by Nernst (1908). The law is expressed as:

$$i \cdot T^{-\frac{1}{2}} = K$$

where i is the current strength, T the duration of the current and K a constant.

In practice this equation does not hold strictly for an amoeba. Hahnert (1932) has found that the value of the constant in Nernst's equation increases with increasing current strength. A constant value of K can, however, be obtained by introducing a modifying factor, itself proportional to -the current strength. Hahnert's equation is:

$$i \cdot T^{-\frac{1}{2}} - (0{\cdot}684\, i) = K.$$

The significance of this modifying factor is unknown. Its value varies for different races of amoebae.

The nature of the shock reaction has been studied by Mast (1931b). An amoeba is negatively galvanotropic, moving to the cathode when placed in an electric field. If at the moment of making the current the amoeba is streaming towards the cathode, there is a marked increase in the rate of streaming. On the other hand, if it is moving to the anode, streaming is retarded and if the current is strong enough the amoeba will reverse the direction of streaming altogether. If the current is very strong, the amoeba disintegrates, this occurring progressively from the anodal side, and continuing as long as the current flows.

The electric current has a solating effect at the cathodal end of the amoeba. This is brought about by the accumulation of positive ions at the cathodal side, union of these ions with hydroxyl ions in the presence of water and migration of the hydrogen ions liberated to the cathode. An increase in the alkalinity of the cathodal end of the amoeba takes place, a condition favouring solation. At the anodal side there is, for similar reasons, an accumulation of negative ions. That these changes do occur can be verified by observations on the epidermal cells of *Tradescantia,* which contain a natural indicator (Mast, 1931b).

The apparatus used was similar to that described by Hahnert (1932). Dimensions of the observation chamber were length 15 mm., width 7·5 mm., depth 4 mm., area of cross section 30 mm^2. The distance between the electrodes was 52 mm. and the potential drop 120 volts. Following Hahnert's usage, current strength was expressed in terms of microamperes per square millimeter of cross section of the observation chamber, one microampere per mm^2. being 1δ. The amoebae were tested in M/100 $CaCl_2$ solution. All reaction times were determined with a stop watch and are given in seconds.

An attempt was made first to determine whether there was any difference between the minimum current densities to which the nucleated and enucleated amoebae respond. Some difficulty was experienced in doing this, for it was soon obvious that the threshold has no definite value. The minimum current producing a response varies for different individuals and even for the same individual when measured at different times. Although this prevents one from assigning definite values to the threshold densities, some differences between the nucleated and enucleated amoebae can be detected. Before discussing the results, it will be necessary to mention the three types of response obtained. Briefly, these are:

1. The amoeba may be moving towards the cathode when stimulated. The response is an acceleration in the rate of streaming, denoted by "A".
2. The amoeba may be inactive. The response is an initiation of streaming towards the cathode denoted by "I".
3. The amoeba may be moving towards the anode. The response taken was the commencement of reversed streaming at the anterior end, denoted by "R".

The results of one experiment are given in Table 2. The type of response obtained after a stimulus is indicated by the letter placed before the reaction time. Each stimulus was separated from the preceding one by a time interval of five minutes, and as soon as the response was obtained the current was immediately broken. The amoeba concerned was bisected one hour previously.

There is one very important point about this case. We have already seen that an enucleated amoeba may not respond to a stimulus because of an inability to move. This may be the case under some conditions of electrical stimulation but it does not always hold. In the above tests,

when the stimuli at times of 60 and 65 minutes were applied, no visible response was detected, although the enucleated amoeba was streaming slowly at the time. As the current density at the time was 10δ, it is possible that the stimulus was insufficiently strong. Even when the density was 13δ, the response from E was irregular. This illustrates well the difference between the irritability of E and N.

TABLE 2.

Time in minutes.	Current density.	Reaction period. E.	Reaction period. N.
0	17	I 1·94 sec.	I 1·97
5	17	I 2·34	A 1·52
10	17	I 2·13	A 1·28
15	17	I 2·30	A 1·41
20	17	I 2·18	A 1·32
25	13	I 2·96	I 2·39
30	13	No effect after 20″	A 1·49
35	13	I 3·60	I 2·00
40	13	I 10·92	A 1·78
45	13	No effect after 20″	R 2·02
50	10	” ” ” ”	R 2·58
55	10	” ” ” ”	R 2·81
60	10	” ” ” ”	R 2·55
65	10	” ” ” ”	R 2·62
70	10	” ” ” ”	R 2·93
75	7	” ” ” ”	R 1·52
80	7	” ” ” ”	R 3·42
85	7	” ” ” ”	R 2·78
90	7	” ” ” ”	R 2·95
95	7	” ” ” ”	A 1·52 (reversed polarity)

When the final stimulus was applied at 95 minutes, N had turned around and was streaming towards the cathode. As far as N was concerned, this had the effect of reversing the polarity and the response was thus of the A type.

That the above experiment did not permanently harm the amoebae was shown by the fact that one hour after being removed from the cell and washed both parts were active, the nucleated portion showing also very firm attachment.

Table 3 gives the results of another experiment using sister halves one day old. In this case the stimuli were separated by 2 minutes instead of 5, and the current density was gradually increased instead of decreased.

TABLE 3.

Time in minutes.	Current density.	Reaction period. E.	Reaction period. N.
0	7	No response within 15 seconds	I 3·00
2	7	” ” ” ” ”	I 3·05
4	10	” ” ” ” ”	I 2·30
6	10	” ” ” ” ”	I 2·19
8	13	” ” ” ” ”	A 1·56
10	13	” ” ” ” ”	I 2·13
12	17	I 8·58	I 1·68
14	17	I 4·67	I 1·49
20	20	I 2·81	I 1·42
22	20	I 4·48	I 1·43
24	23	No effect within 10 seconds	I 1·50
26	23	” ” ” ” ”	I 1·55

The long interval between 14 and 20 minutes was made owing to the fact that during this interval the nucleated fragment put out pseudopodia in several directions, rendering it difficult to observe the response. Although the enucleated fragment showed responses at 17δ and 20δ, for some reason no response was obtained at 23δ, but movement as a whole to the anode took place, indicating a negative surface charge. A nucleated amoeba, when unattached, also moves as a whole to the anode.

A third example of this type of experiment is given in Table 4 and shows the great variation in the apparent threshold density for enucleated amoeba. The amoebae concerned are the same as used in the previous experiment (see Table 3), and were tested 36 hours after the conclusion of that experiment.

TABLE 4.

Time in minutes.	Current density.	Reaction period. E.	Reaction period. N.
0	1	No response within 10 seconds	No response within 10 seconds
4	1·5	" " " " "	A 4·32
8	2	" " " " "	I 5·22
12	2·5	" " " " "	A 3·83
16	3	" " " " "	A 3·02
20	3·5	" " " " "	I 4·16
24	3·5	I 8·38	I 3·85
28	3·5	No response within 10 seconds	A 3·58
32	3·5	" " " " "	A 3·90
36	7	I 4·90	I 3·17
40	7	I 4·47	I 3·23
44	10	No response within 10 seconds	I 3·18
48	13	I 7·58	I 3·10
52	17	I 6·27	I 2·93

The threshold density for the enucleated amoeba is appreciably higher than that for the control half. Further, if the irregularity in the response of the enucleated amoeba is the result of fatigue, it is interesting that no similar irregularity was found in the behaviour of the nucleated sister half. Either E cannot recover readily from the effects of the stimulus, i.e. disturbance of the intracellular distribution of the positive and negative ions, or else it cannot maintain locomotion for any great length of time. I think the second view is probably the correct one.

An interesting feature shown by both types of amoebae is that the reaction times for oft-repeated stimuli sometimes appear to be cumulative. This is shown in the figures of Table 5. In all the tests, the current density was constant at 33δ. Each stimulus was separated from the preceding one by an interval of 15 seconds as timed from the commencement of that stimulus; i.e. to find the interval during which no current was passing, it is necessary to subtract the preceding reaction time from 15 seconds. The tests were made on five pairs of amoebae twenty-four hours after they had been bisected. In order to obtain the same type of response from both E and N during any one experiment it was necessary to wait until both were in similar states of either activity or rest. The difficulty of obtaining inactive nucleated amoebae for examining the "I" type response was overcome by gently agitating the amoeba in a pipette until it became inactive.

TABLE 5.

Number of experiment.	Type of response.	Reaction times in seconds.
1	A	N 2·20, 1·86, 1·02, 1·29 E 1·91, 1·73, 0·82
2	A	N 1·31, 1·28, 1·46, 1·25 E 1·92, 1·59, 1·28
3	I	N 2·3, 2·19, 1·58 E 2·95, 2·50, 2·28
4	I	N 2·1, 1·88, 1·02, 1·74 E 1·72, 2·19, 1·43
5	R	N 6·96, 6·02, 2·52, 2·05 E 4·46, 1·85, 2·85, 3·42

Although the reaction times indicate a cumulative effect in both cases, it should be emphasized that the figures for the enucleated amoebae are by no means typical. They were actually selected from a whole series of experiments merely in order to demonstrate that the phenomenon can be observed in enucleated amoebae. Although a constant characteristic of nucleated amoebae, the cumulative effect was observed in only 5 out of 28 enucleated amoebae. The failure to observe the effect may again be connected in some way with the degree of activity of the amoeba.

The effect of a current of constant strength over an extended period has been studied for the case of normal amoebae by Hahnert. The only apparent effect of the current is on the rate of locomotion. This shows an initial rise but is eventually retarded. The degree of the effect is related to the current density. Due to the discontinuity of locomotion shown by the enucleated amoeba, it has not proved possible to observe this effect. In some cases an initial rise was detected, so it seems probable that the fall would also be observed provided a sufficiently active amoeba were available.

DISCUSSION.

From the foregoing account, we can conclude that normal responses to stimuli are obtainable from enucleate amoeba cytoplasm and this is in harmony with the findings of Verworn (1890), Balbiani (1888) and Jennings and Jamieson (1904), all of whom found normal behaviour in enucleated fragments of Ciliate Protozoa. Failure to obtain a response from an enucleated amoeba is generally related to the inactivity of the amoeba at the time of stimulation. Pantin (1930) has shown that amoeboid movement consists of an anaerobic phase, followed by an aerobic phase, in which the products of the first phase are oxidized. As the cyanide-sensitive respiration of an amoeba is abolished 24 hours after enucleation (Clark, 1942), it is highly probable that the inactivity of the enucleated amoeba is due to a breakdown of the second, recovery phase. This would explain why an enucleated amoeba does not always give a locomotory response, as the accumulation of the reaction products of the anaerobic phase prohibits any further streaming until those products have been removed in some manner. Removal by oxidation through a cyanide-sensitive mechanism is excluded. Diffusion processes may be involved, and if they are, it would be possible to explain why the amoeba will give normal responses to several stimuli and will then pass into an inactive condition, from which it cannot be drawn by powerful and even destructive stimuli. Enucleated amoebae become quite active when heated slowly to about 26° C., but generally lapse into inactivity again on cooling. Are the diffusion or other processes responsible for removing the metabolites accelerated sufficiently by the heat to enable the amoebae to show greater activity? Furthermore, frequently transferring an enucleated amoeba to fresh droplets of distilled water greatly increases its activity. Is it permissible to draw the naive conclusion that the metabolites are being washed away more rapidly, or is the effect solely an osmotic one, entrance of the distilled water into the amoeba increasing its turgidity, hence its activity. It is hoped that further work will supply answers to these interesting questions.

SUMMARY.

Under favourable conditions, normal responses can be obtained from enucleated amoebae towards contact, agitation, heat and electricity.

Although a normal shock reaction to light can be obtained, an enucleated amoeba fails to orient in a horizontal beam.

The ability of the amoeba to move and show attachment to the substrate at the time of stimulation are the most important factors determining whether a response will be obtained or not.

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A NOTE ON A LYTIC PHENOMENON SHOWN BY GROUP B STREPTOCOCCI

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In a recent outbreak of Scarlet Fever in a country district it was suspected that the milk was the vehicle of infection. Five samples of the milk supplied to the area were submitted to examination for haemolytic streptococci. After centrifugation, the mixed cream and sediment were stroked out on the surface of sheep blood agar plates which were incubated over-night at 37° C. From three of the samples streptococci were obtained which were apparently haemolytic on the primary plates but non-haemolytic on sub-culture.

Examination of the primary plates revealed the presence of many staphylococci of the $\alpha\beta$ type, that is, their colonies were in the centre of a clear zone, which in turn was surrounded by a large darkened zone, where the staphylococcal β toxin had altered, but not lysed, the sheep red cells. Wherever there were colonies of the streptococcus within these zones of darkening, the colonies were surrounded by an area of complete haemolysis, whilst elsewhere on the plates they produced no distinct haemolysis.

To confirm this observation, a colony of these staphylococci was grown by spot-inoculation on the centre of a sheep blood agar plate. After 24 hours' incubation the streptococci were inoculated on to the darkened zone. Within two hours haemolysis was visible around the streptococcal growth.

In the case of a staphylococcus producing α toxin only and giving therefore an area of partial haemolysis around a zone of clear haemolysis no variation of the haemolysis was caused by growing the streptococcus in or near the zone of partial haemolysis, but in the dark zone around staphylococci producing β toxin only, haemolysis was readily produced.

When the streptococcus was grown near the edge of one of these uniform dark zones, clearing of the medium could be seen at the point nearest to the streptococcus colony, suggesting that an invisible agent, or the products of such an agent, had reached the red cells already altered by the staphylococcal β toxin (Fig. 1).

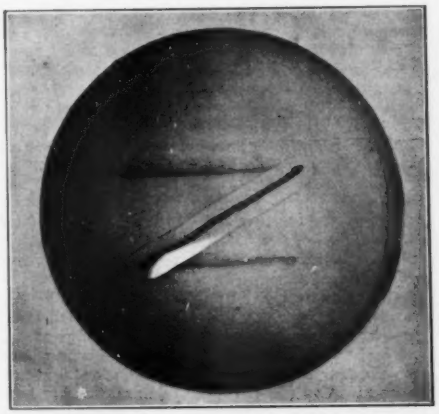

Fig. 1. The central streak is due to growth of staphylococci on sheep blood agar. The zone around it is due to β toxin. The lower streak shows a growth of Group B streptococci with haemolysis where it adjoins the staphylococcal zone. The upper streak shows growth of non-Group B streptococci.

Under anaerobic conditions the effect was more marked, and definite pitting of the medium could be seen in position coinciding with the clearing, indicating that some constituent or constituents of the medium had been altered to give diffusible substances. The pitting was too deep to be due entirely to migration of the haemoglobin liberated by red cell lysis. Furthermore, the clear area was glassy-clear and not faintly turbid, as is usually found when there is cell lysis only.

To demonstrate the lysis, sheep and ox blood were found suitable. No lysis was obtained when horse, human, rabbit or guinea-pig blood was used.

A number of strains of streptococci were then tested. Of 55 from various human sources (throat, sputum, urine, skin, tooth and nose), some haemolytic, others non-haemolytic, and

5 non-haemolytic anaerobic strains of human origin (received through the courtesy of Miss H. Butler), none gave the reaction when grown with the staphylococcus, although some showed a narrow area of incomplete lysis. Forty-three strains from cases of suspected and nineteen from cases of definite bovine mastitis were tested. Of these, all but three gave the reaction. Serological examination then revealed that of all the strains tested only those which belonged to Group B Lancefield gave the reaction, and none belonging to Group B failed to give it. Five Group B strains of human origin (tonsils, vagina and skin lesion) also gave the positive reaction. Three representative strains of each of the Groups A, C and G were tested. None was positive. Where normal haemolysis occurred, as with the Group A strains, the haemolytic area was no more extensive where it overlapped the staphylococcal zone than elsewhere in the medium. To determine whether lysis, similar to that caused by non-haemolytic Group B strains, was occurring but being masked by the ordinary streptococcal haemolysis, the boiled broth technique, described later, was used. The results were still negative. No Group B strain produced sufficient lysis by itself to interfere with the reading of the haemolysis induced within the staphylococcal zone.

The presence of the active agent in the solid medium could be detected fully half a centimetre from the streptococcal colonies. There was no detectable difference in the degree to which the separate strains produced the agent, although 13 serological sub-types were represented in the strains tested.

It is known that certain non-haemolytic staphylococci, when grown on blood agar near a staphylococcus with a zone of β toxin, produce lysis within this zone just as do the Group B streptococci (Christie and Graydon, 1941). Some evidence has been given to show that the active agent from the staphylococcus is staphylococcal lipase. Group B and other streptococci were therefore tested for lipolytic activity by growing on cream blood agar (Orcutt and Howe, 1922) and on a butter-fat neutral red agar, but none could be detected.

Many non-haemolytic air-borne organisms are known to cause lysis around β haemolytic staphylococci but whereas these contaminants also cause similar lysis in the medium close to colonies of *Cl. welchii*, the Group B streptococci do not do so. Furthermore, the contaminants near the staphylococcal colonies produce a haemolytic zone with a sharply defined edge, whereas the streptococci produce a zone with an indefinite edge. Thus, there must be at least three bacterial agents capable of causing lysis of red cells which have been treated with staphylococcal β toxin, namely those from Group B streptococci, and from non-haemolytic staphylococci (which are not identical), and that from some air-borne contaminants (which again acts in a different manner).

Group B streptococci are known to be non-fibrinolytic. Eight strains producing the above lysis (3 of animal and 5 of human origin) were tested against human fibrin and found to be non-fibrinolytic. A strongly fibrinolytic streptococcus, not of Group B, was unable to induce the above-mentioned lysis. Fibrinolysin was therefore ruled out as the responsible lytic agent.

Since Group B streptococci commonly occur in milk, take part in a reaction of an apparently digestive nature, and are not lipolytic, their ability to digest casein was examined in an unsuccessful effort to find a test for the lytic agent other than the above test with sheep cells. They were grown on agar rendered slightly turbid with fat-free milk (4 p.c.), and they showed only faint traces of clearing, much less than was shown by many streptococci of other serological groups; e.g. *Str. faecalis*; which does not produce the haemolytic reaction, gave large clear zones, its digestive power for the casein being visible after two hours.

A strain of Group B streptococci was grown in meat infusion broth for 18 hours and the broth filtered through a Seitz EK pad. Tests proved the filtrate to be sterile. The initial pH of the broth was 7·6 and the final pH 7·0. A drop of the filtrate on the dark zone around a staphylococcus colony caused lysis, just visible after 10 minutes and quite definite after one hour. The clearing was most complete at the edge of the zone. Readjustment of the pH of the filtrate to 7·6 had no effect on the amount of clearing it could produce.

Heating of the filtrate at 56° C. for 30 minutes, or at 60° C. for 10 minutes, altered it so that it produced only a trace of lysis and further heating at 56° C. and 60° C. for 30 minutes had no more effect. On the other hand, a sample heated at 100° C. for 5 minutes gave almost as strong a reaction as the unheated filtrate. Heated and unheated fresh broth gave no reaction. The unexpected positive result with filtrate heated at 100° C. suggested that the samples, partly inactivated by heat at 56° C. or 60° C., would be re-activated by heating to 100° C. On testing, this was found to be the case. The test was repeated with two other Group B strains, with similar results.

Landsteiner and von Rauchenbichler (1909), using crude staphylococcal α toxin, found that this was inactivated when heated to 65° C. for 30 minutes, with reactivation to one-quarter of the original titre after five minutes' further heating at 100° C. The inactivation at 65° C. was due, not to destruction of the toxin, but to the combination of the toxin with some constituent of the broth, a combination that was disrupted by heating at 100° C. This phenomenon seems to be related to that found with the streptococcal filtrate mentioned above.

It was found possible to reproduce these findings in test-tube titrations, using Seitz-filtered staphylococcal β toxin and the streptococcal broth filtrate. Diluted samples of the filtrate were added to constant volumes of the β toxin, and then washed sheep red cells were added. After

incubation for one hour at 37° C., the cells were found to be lysed in the tube containing the undiluted filtrate, but the power of the filtrate to induce lysis was quickly lost on dilution. An undiluted sample which had been heated at 56° C. for 30 minutes caused only a trace of lysis, but a sample heated at 56° C. for 30 minutes and then maintained at 100° C. for five minutes, had a lytic potency similar to that of the unheated filtrate. A sample heated for five minutes at 100° C. was as potent as the raw filtrate (see Table 1). When an undiluted sample of the filtrate was allowed to act on the red cells for 30 minutes at 37° C., lysis occurred immediately on addition of the β toxin.

TABLE 1.

Results of haemolysis titrations, using streptococcal filtrate, staphylococcal β toxin and sheep red cells.

Series I. Ten drops of each diluted preparation of streptococcal filtrate were added to the corresponding tube. Ten drops of β toxin (raw, filtered toxin diluted 1: 10 with normal saline) were added to each tube. Two drops of a 10 p.c. suspension in saline of washed sheep red cells were then added and the tubes incubated at 37° C. for 1 hour.

Series II. As above, except that the filtrate was first heated to 56° C. for 30 minutes.

Series III. As in series I, except that the filtrate was first heated to 56° C. for 30 minutes, and then to 100° C. for five minutes.

Series IV. As in series I, except that the filtrate was first heated to 100° C. for five minutes.

Series V. As in series I, but with saline substituted for the β toxin.

Series VI. As in series I, but with saline substituted for the streptococcal filtrate.

	Initial dilution of filtrate.				
	10/10	8/10	6/10	4/10	2/10
Series I	++++	++++	++++	++++	+
Series II	+	+	+	—	—
Series III	++++	++++	++++	+	—
Series IV	++++	++++	++++	++++	+
Series V	—	—	—	—	—
Series VI	—	—	—	—	—

++++ Indicates complete lysis. + Indicates 25 p.c. lysis. — Indicates no lysis.

The quantity of the agent present in the filtrate was not found to be increased by incubation for longer than 18 hours.

The staphylococci most commonly found in animals are predominantly β toxin-producers (Minett, 1936; Cowan, 1938). It is only with these staphylococci that Group B streptococci produce the lysis described above. Not infrequently staphylococci and Group B streptococci are found together in the bovine udder; there may be some significant connection between this occurrence and the fact that together they have a lytic power which neither has independently.

The results obtained with the Group B streptococci of human origin give further evidence of the close relationship of the animal and human strains of this group, in spite of the differences in serological type and in some other characteristics established by Simmons and Keogh (1940).

The negative results obtained when horse blood was substituted for sheep blood suggest that in routine tests for haemolytic streptococci, horse blood only should be used, particularly where β toxin-producing staphylococci are to be expected, as in milk samples.

The number of members of the various groups tested was not large, but the results obtained so far indicate that the phenomenon may provide a relatively simple confirmatory or substitute test for Group B streptococci.

SUMMARY.

Strains of Group B streptococci, of animal and human origin, produce an agent which will lyse sheep and ox, but not human, horse, rabbit or guinea-pig red cells, when these cells have been altered by staphylococcal β toxin.

The agent is extracellular, filtrable, and thermostable.

Streptococci of human and animal origin, belonging to Groups other than Group B, have not been found to produce this agent.

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THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA. 2.

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Pursuing our study (Atkinson and Woodroofe, 1944) of the types of Salmonella occurring in Australia, we have typed a further group of cultures[2]. Among them we found another strain of the new type *S. adelaide* (Atkinson, 1943) of which only one had so far appeared, four strains of *S. blegdam* which, as far as we are aware, has only once been found before by Kauffmann (see Topley and Wilson, 1936), one strain each of *S. newport* and *S. new-brunswick* which have not been recorded in Australia before. Besides these known types we have also found two strains of a new serological type closely related to *S. reading* and for which the name *S. kensington* is suggested.

ANTIGENIC ANALYSIS OF CULTURES.

The methods used for this work have been described in our previous paper.

We encountered a variety of types, namely *S. typhi-murium*, *S. cholerae-suis* var. *kunzendorf*, *S. newport*, *S. bovis-morbificans*, *S. blegdam*, *S. new-brunswick*, *S. adelaide* and the new type *S. kensington*.

The strains of *S. typhi-murium* were mainly IV, V types but a few I, IV, V types and IV-variants with or without I occurred.

Many strains agglutinated to a high dilution with H antiserum prepared against *S. heidelberg*. This serum was absorbed with *S. typhi-murium* after which it was specific for the H antigen r and gave no agglutination with *S. typhi-murium*. A test with this serum was necessary to differentiate with certainty between the IV, V type of *S. typhi-murium* and *S. heidelberg* which differ only in their specific H antigens.

The culture identified as *S. cholerae-suis* var. *kunzendorf* contained only group H antigen and could not be forced into the specific phase by any of the usual procedures; it also produced abundant H_2S, another reaction which distinguished it from the diphasic type. Its O antigens were recognized by agglutination with specific O antisera.

S. newport was distinguished from *S. kottbus* by its group H antigens; it did not contain antigen 5 but contained antigens 2 and 3.

The strains of *S. bovis-morbificans* were identified by their O antigen VIII, their specific H antigen r and their group H antigen 5.

The strains of *S. blegdam* contained O antigen IX, H antigen g and agglutinated with H antisera specific for the factors m and q; they did not agglutinate with H antisera specific for the factors f, p and u.

In tests for O antigens the strain identified as *S. new-brunswick* fell into Group E of the Kauffmann-White schema but did not agglutinate with O antisera produced against *S. minnesota* (XXI, XXVI). It belonged therefore to the second part of Group E characterized by O antigen XV, and agglutinated with an O antiserum specific for XV. It was diphasic possessing H antigens lv in Phase 1; the presence of v was confirmed by agglutination with a specific v serum.

S. adelaide was readily recognized by its new O antigen XXXV and its H antigens fg. This strain also completely absorbed the agglutinins from *S. adelaide* antiserum.

The new type *S. kensington* contained the O and specific H antigens of *S. reading* but did not contain group H factor 5. A detailed description of the antigen analysis of *S. kensington* will be given in a separate paper.

THE ORIGIN AND FREQUENCY OF OCCURRENCE OF THE SALMONELLA TYPES.

The group of cultures recorded here comprised 41 strains of which 25 were *S. typhi-murium*, 6 *S. bovis-morbificans*, 4 *S. blegdam*, one each of *S. adelaide*, *S. new-brunswick*, *S. newport* and *S. cholerae-suis* var. *kunzendorf*, and 2 *S. kensington*.

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[1] Working with a grant from the University of Adelaide.

[2] We wish to thank all those who have kindly supplied cultures.

The majority of strains of *S. typhi-murium* came from faeces of children with gastro-enteritis. All strains were tested in Bitter medium for fermentation of maltose, xylose and rhamnose; the results are given in Table 1.

TABLE 1.

Source and reactions of strains of S. typhi-murium.

Source of strain.	Number of strains found.	Bitter test. Maltose.	Xylose.	Rhamnose.	Somatic antigens.
Children with gastro-enteritis	2	±	—	+	I, IV, V
	2	—	—	—	I, IV, V
	2	—	—	—	IV, V
	1	±	—	—	IV, V
	4	—	—	+	IV, V
	5	±	—	+	IV, V
	1	±	—	±	IV, V
	2	+	—	+	IV, V
	1	—	—	+	I, IV
	1	—	—	+	IV
	1	±	—	+	IV
Adults with gastro-enteritis	1	+	—	+	IV, V
	1	+	+	+	IV, V
Guinea-pig with epidemic disease	1	+	±	+	IV, V

The strains previously reported were mainly IV-variants which were rare in the present group. Our previous group gave many positive Bitter tests with all three substrates whereas the present group showed many negative Bitter tests particularly with xylose. Edwards and Bruner (1940) found their IV-variants to be poor fermenters compared with their full antigenic types. Our results contrast with these findings.

Four of our strains of *S. bovis-morbificans* came from faeces of two children and two adults suffering from gastro-enteritis; the other two strains were found in kidneys taken from two sheep condemned at the abattoirs as unsuitable to be used for meat.

The four strains of *S. blegdam* were isolated from blood and faeces of four military patients with enteric-like fevers; three strains were isolated in New Guinea: the other strain was isolated in Adelaide from a man who had just returned from New Guinea.

The strain *S. adelaide*, the second example of this new type to be found, was isolated on six occasions from the faeces of an infant with gastro-enteritis. The two strains of the new type *S. kensington* and the single strains of *S. new-brunswick* and *S. newport* came from the faeces of children with gastro-enteritis.

The one culture of *S. cholerae-suis* var. *kunzendorf* was isolated from synovial fluid from an arthritic knee-joint in an adult.

In Table 2 a summary is given of the observations recorded in this paper.

TABLE 2.

Salmonellas described in this paper.

Salmonella.	Total number of strains.	Where isolated.	Source.	Number isolated.	Type of illness.
S. typhi-murium	25	South Australia	Children (faeces)	22	gastro-enteritis
			Adults (faeces)	2	gastro-enteritis
		Queensland	Guinea-pig	1	epidemic disease
S. bovis-morbificans	6	South Australia	Children (faeces)	2	gastro-enteritis
			Sheep kidney	2	septicaemia
		New South Wales	Adults (faeces)	2	gastro-enteritis
S. blegdam	4	South Australia	Adult (blood and faeces)	1	enteric-like fever
		New Guinea	Adult (blood and faeces)	3	enteric-like fever
S. kensington	2	South Australia	Children (faeces)	2	gastro-enteritis
S. adelaide	1	South Australia	Child (faeces)	1	gastro-enteritis
S. new-brunswick	1	South Australia	Child (faeces)	1	gastro-enteritis
S. newport	1	South Australia	Child (faeces)	1	gastro-enteritis
S. cholerae-suis var. *kunzendorf*	1	South Australia	Adult (synovial fluid from knee)	1	arthritis

DISCUSSION.

In contrast with our previous findings on the predominating Salmonella, *S. typhi-murium*, we found few IV-variants among the 25 additional strains recorded here. Although they were mainly full antigenic types all but two of them gave a negative reaction in Bitter xylose and many gave a weak or negative reaction in Bitter maltose. Our IV-variants previously described gave mainly positive Bitter tests. These results differ from those of Edwards and Bruner (1940) who found, among cultures from animals, many positive Bitter tests with the full antigenic type, but few positive Bitter tests, particularly in xylose and maltose, with IV-variants. The sources of our cultures differ from those of Edwards and Bruner and may account for the different results, the significance of which cannot be assessed until many more Australian strains from various sources have been investigated.

S. bovis-morbificans has previously been found in Australia in sheep and occasionally in pigs but no human strains have been recorded. We obtained four strains from faeces of adults and children with gastro-enteritis and two strains from the kidneys of sheep. In the United States of America Bornstein (1943) found only one culture of *S. bovis-morbificans* out of 500 Salmonellas isolated from man, and Edwards and Bruner (1943) found none of this type in 3,090 cultures of animal or human origin. Apparently in Australia this organism frequently infects sheep; as it can cause gastro-enteritis in man and may often be present in sheep, the need for adequate meat inspection and the thorough cooking of mutton is clearly indicated.

The occurrence of *S. blegdam* in the blood and faeces in enteric-like fever contracted in New Guinea is especially interesting. As far as we can discover this organism has only been found on one previous occasion. All our cases occurred in individuals receiving routine inoculations of T.A.B. vaccine, which in spite of its content of O antigen similar to that of *S. blegdam* failed to protect against severe infection with this organism. This result directly contrasts with that which we previously recorded for infection with *S. paratyphi B* which was mild in inoculated individuals but proved fatal in the one uninoculated person involved in the epidemic.

We were very pleased to find another strain of *S. adelaide*, of which only one had so far appeared. Just after we had identified this culture we received from Dr. P. R. Edwards, of the University of Kentucky, a letter informing us that Dr. J. Taylor, of the Emergency Public Health Laboratory Service of Great Britain had sent him an untyped culture which proved to be identical with *S. adelaide*. Dr. Taylor had three additional strains of the same type. This recognition of *S. adelaide* in Great Britain suggests that it may not be an uncommon type and may be widely distributed; it is clearly distinguished from other types by its new O antigen, for which the number XXXV has been suggested.

S. newport has frequently been found in the United States of America in both man and animals; we have found only one human strain here.

S. new-brunswick. (Edwards, 1937), occurs in the United States mainly in fowls and occasionally in ruminants and swine. Edwards and Bruner (1943) found among their 3,090 Salmonellas, 43 strains of which 39 came from fowls; there were no human strains. Bornstein (1943) in a list of Salmonellas and their occurrence indicates that no more than one or two human strains have ever been found. On account of the rarity of human infections our recovery of a strain from a case of gastro-enteritis in a child is particularly interesting.

S. choleraе-suis var. *kunzendorf* is commonly found in many parts of the world, including Australia. In the United States Edwards and Bruner found 329 strains among their 3,090 cultures; it was one of the commoner types occurring

in man and appeared ten times as often as the diphasic type; in animals it was found chiefly in swine and carnivores; many of the human strains were isolated from blood. Our single strain came from the arthritic knee-joint of an adult who gave no previous history of diarrhoea, vomiting or fever. Bornstein quotes cases of arthritis in infants due to this organism.

We will not discuss the new type *S. kensington* here, as a separate report on these cultures is being prepared.

The outstanding feature of our results is the discovery, among such a small collection of Salmonellas, of so many unusual types, including a new one.

SUMMARY.

Forty-one strains of Salmonellas isolated in Australia have been typed serologically.

S. typhi-murium occurred most frequently and few IV-variants were found.

A new serological type, for which the name *S. kensington* is suggested, was discovered.

The remaining strains were identified as *S. adelaide, S. blegdam, S. newbrunswick, S. newport, S. bovis-morbificans* or *S. cholerae-suis* var. *kunzendorf*.

Most of these Salmonellas came from faeces of human cases of gastro-enteritis or enteric-like fever.

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THE GROWTH OF PSITTACOSIS VIRUS IN THE ALLANTOIC CAVITY OF THE DEVELOPING EGG

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The virus of psittacosis was first shown to multiply in the developing egg by Burnet and Rountree (1935), who demonstrated the ready propagation of avian strains on the chorioallantoic membrane. Using more virulent strains of both human and avian origin, Lazarus and Meyer (1939) have also given a full description of the chorioallantoic growth of the virus and have adapted it to the production of large quantities of virus for serological investigations. Infection by the amniotic route was described by Burnet and Foley (1941), who found that multiplication occurred in the embryonic respiratory tract. The yolk-sac method of inoculation was successfully used for the cultivation of psittacosis virus by Yanamura and Meyer (1941).

The present study was made following the observation that a recently isolated avian strain gave an abundant growth when inoculated into the allantoic cavity of developing eggs.

MATERIALS AND METHODS.

The strain used in the majority of experiments was isolated from a naturally-occurring outbreak in a breeder's stock of Gouldian finches. Isolation of psittacosis virus has previously been reported from finches of this type (Tremain, 1938). The present epizootic was highly fatal, killing off the entire stock of some 50 birds within a month. A strain of virus was readily isolated from the spleens of two birds by inoculation of mice intranasally and on to the chorioallantoic membrane of eggs. It proved to be of high virulence for mice by both intraperitoneal and intranasal routes, smears of infected tissues showing typical L.C.L. bodies in large numbers.

The other strains tested were two isolated from pigeons and maintained for several passages in mice, two of human origin isolated some years ago and stored after passages in both eggs and mice, and a further human strain recently isolated by Mrs. McEwin of the Institute of Medical and Veterinary Science, Adelaide. Two strains of lymphogranuloma inguinale virus, also tested, were isolated 2 years ago from human gland lesions.

Titrations in mice were made by intranasal inoculation, according to the method described by Rudd and Burnet (1941). Allantoic inoculation of fertile eggs was made through a small drill-hole in the shell, and 0·25 c.c. used as a standard inoculum. The eggs were incubated at a temperature of 37° C. before inoculation and 35° C. after inoculation. Smears of egg fluids and tissues were stained by Castaneda's method to provide a ready identification of L.C.L. bodies. All fluids were cultured on blood agar and in nutrient broth to ensure absence of bacterial contamination.

PROPERTIES OF INFECTED ALLANTOIC FLUID.

Inoculation of virus suspensions into the allantoic cavity gave rise to specific changes in the fluid. The following description has been taken from observations on 10 day eggs inoculated with the avian strain "Gould", which was maintained for twelve successive allantoic passages in eggs. Three days after inoculation the fluid was perceptibly opalescent and contained fragments of tissue debris. Microscopically a considerable number of L.C.L. bodies could be seen dispersed throughout the fluid, with large clumps and "microcolonies" adhering to tissue fragments. As incubation proceeded the fluid became more turbid and smears showed increasing numbers of elementary bodies. At the same time it became somewhat mucoid in consistency and often contained large stringy clots of urates and cell debris. Death occurred, as a rule, on the fifth or sixth day. Smears of fluid made at the time of death showed many thousands of extracellular clumped and dispersed L.C.L. bodies in each oil immersion field, while the mucoid clots showed even greater numbers. The production of excessive quantities of mucoid material in fluids ap-

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peared to be directly associated with psittacosis infection, although some mucus was frequently present in uninoculated eggs of corresponding age.

The amount of fluid harvested varied with the age of the eggs. With young eggs, e.g. 7 days at inoculation, it was found that death occurred more rapidly with a smaller production of virus than in older eggs. With 12 or 13 day eggs the incubation period was extended but the amount of fluid present at death was small, and so mucoid as to present difficulties in removing it aseptically from the egg. An experiment involving eggs of different ages which records these details is given in Table 1. All eggs received the same inoculum of undiluted allantoic fluid, and were incubated under the same conditions. It has been found that 9 or 10 day eggs give the most satisfactory results as regards the quantity and quality of fluid harvested and also produce a maximum multiplication of virus.

TABLE 1.

Inoculation of eggs at different ages.

	Age before inoculation.				
	7 days.	8 days.	9 days.	10 days.	11 days.
No. inoculated	6	6	5	6	6
Days of survival (average)	3·3	4·0	5·2	6·2	6·0
Mucus present	—	—	±	+	+
Quantity of fluid obtained, average (c.c.)	1·7	6·2	8·2	7·3	4·6
Smear of fluid	++	+++	++++	++++	++++

Macroscopically, no visible focal or generalized changes could be observed in the membranes lining the allantoic cavity, although direct smears showed that large numbers of cells containing L.C.L. bodies were to be found in all parts of the membranes.

INFECTIVE TITRE OF VIRUS IN ALLANTOIC FLUID.

A titration of the infectivity of allantoic fluid is shown in Table 2. In this experiment, three groups of 10 day eggs were inoculated with the same virus suspension (fresh undiluted allantoic fluid) by the allantoic, yolk-sac, and chorioallantoic routes respectively, there being six eggs in each group. When moribund or just dead, which in the case of the yolk-sac and chorioallantoic inoculations was on the fourth day, and of the allantoic inoculations the sixth day, the eggs were opened and the infected tissues or fluids collected and pooled. Serial ten-fold dilutions of allantoic fluid and of 10 p.c. suspensions of ground yolk-sac or chorioallantoic membranes were inoculated intranasally into three series of mice, the inoculum being 0·05 c.c. After 7 days the mice were killed and the lungs examined for foci of consolidation. It can be seen that the titre of the allantoic fluid group is somewhat higher than that for either of the other groups. This high figure for allantoic fluid was remarkably constant for a number of titrations done in this way, and indicates that there are at least 10^9 infective doses per c.c. of fluid.

TABLE 2.

Titration in mice of virus grown in different tissues in eggs.

Virus dilutions.	C.-A. membrane suspension.	Yolk-sac suspension.	Allantoic fluid.
10^{-5}	—	0, 0, 0	0, 0, 0
10^{-6}	0, 0, 0	0, 0, 0	6, 4, 4
10^{-7}	0, 1, 0	1, 3, 4	39, 30, 1c
10^{-8}	0, 4, 2	15, 9, 18	2c, 2c, 2c
10^{-9}	21, 35, 29	—	—

Figures represent focal lesions in mouse lungs. 1c, 2c, represent degrees of lung consolidation, 4c being complete consolidation of both lungs.

Infected allantoic fluid was also titrated allantoically in eggs to determine the sensitivity of this route to small doses of virus. Serial ten-fold dilutions of pooled freshly harvested fluids were inoculated into 9 day eggs and into mice, the eggs receiving 0·25 c.c. allantoically, and mice 0·05 c.c. intranasally. After a further period of nine days incubation the eggs were opened and the fluids examined by smear for the presence of virus. All fluids from the 10^{-10} and 10^{-9} series were inoculated intranasally into mice as a further test for the virus. Three mice were used for each sample, and, if still alive, were killed 7 days later and examined for evidence of lung consolidation and L.C.L. bodies. The results are set out in Table 3. This shows again that the

amount of viable virus in the original fluid was large and even in high dilution was still capable of infecting eggs by this route. The difference in titre between eggs and mice in this series is largely explained by the five-fold greater inoculum which the eggs received. The degree of multiplication in the eggs receiving limiting dilutions was not maximal in the time allowed for incubation and several cases showed a negative smear although subsequent mouse tests disclosed a significant amount of virus.

TABLE 3.

Comparative titration of allantoic fluid in eggs and mice.

Dilution of fluid.	Eggs.	Mice.
10^{-2}	0, 0, 0, 0, 0, M	—
10^{-3}	0, 0, M, M, S, S	0, 0, 0, 0
10^{-4}	+, +, +, +, +, +	1, 1, 2, 3
10^{-7}	+, +, +, +, +, +	6, 17, 42, 33

0 = No L.C.L. bodies seen in fluid and mouse test negative.
M = No L.C.L. bodies seen in fluid and mouse test positive.
S = L.C.L. bodies seen in smear and mouse test positive.
+ = Large numbers of L.C.L. bodies seen.

GROWTH OF OTHER PSITTACOSIS STRAINS.

Five further strains of psittacosis tested in eggs by the allantoic route of inoculation all gave results similar to those described above for the strain "Gould". Two pigeon strains which had a low virulence for mice and which had not previously been passaged in eggs required two or three successive allantoic passages to develop maximal virus multiplication. Three strains isolated from human cases grew abundantly at the first allantoic inoculation. No difference in growth characteristics or morphological features could be detected in any of the strains examined.

Efforts were also made to obtain an allantoic multiplication of lymphogranuloma inguinale virus. Two strains were tested which had proved difficult to maintain by the yolk sac method, yielding in this site a meagre and irregular growth which did not allow the effective production of a complement-fixing or Frei antigen. In spite of several attempts it has not yet been found possible to establish this virus in the allantoic cavity.

Samples of fluid from eggs infected with each of the six psittacosis strains were examined for their antigenic properties in complement fixation tests. Unfortunately the only sera available were obtained from convalescent lymphogranuloma inguinale cases two years ago, and at that time were shown in this laboratory to react satisfactorily with yolk-sac and mouse-lung preparations of psittacosis virus in complement fixation tests. These sera gave fixation against allantoic fluid antigens of all six strains although minor differences of titre were found. The highest dilution of serum found to give complete fixation was 1: 32 to 1: 64, a titre somewhat lower than that given when the sera were fresh.

DISCUSSION.

Cultivation of influenza virus by the allantoic method has already been widely adopted for many laboratory procedures and for the preparation of vaccines. The growth of psittacosis in this site provides a further method of isolating and obtaining the virus in large quantities. Already many techniques have been described which permit the study of this virus in the laboratory, utilizing animals, eggs and tissue cultures. From the point of view of safety, cheapness and ease of handling the egg offers many advantages, and of the various techniques of inoculation in the egg, the allantoic is perhaps the one of choice. The manipulations and apparatus required are reduced to a minimum as regards both aseptic transfer and harvesting of the virus. Samples of fluid may be removed from the egg without affecting the viability of the embryo or the development of infection, which may thus be observed at intervals as a continuous process in one egg.

Allantoic fluids from eggs infected with virus provide a very suitable reagent for serological tests. In 1941, Australian and American workers almost simultaneously showed the suitability of allantoic fluid as a complement-fixing antigen in the case of influenza virus. Beveridge and Burnet (1944) have also successfully used influenza virus fluids for demonstrating a specific skin test related to circulating influenza antibodies. These fluids contain virus particles which have collected

as a result of the disruption of infected cells in the lining membranes, together with any specific soluble products which have been formed during growth. Extraneous soluble and insoluble protein materials are reduced to a minimum. In the case of psittacosis virus, further purification by centrifugation can be undertaken if required.

The sensitivity of the allantoic route to very small infecting doses of psittacosis virus would suggest that it is also suitable for primary isolation from suspected cases. No opportunity has yet occurred to test it in this way, although some strains successfully cultivated had been previously maintained only in mice. The failure to grow the virus of lymphogranuloma inguinale in the allantoic cavity indicates a specificity of growth requirements which may perhaps be compared with the failure of lymphogranuloma to infect mice intraperitoneally. This latter property is usually taken as the chief biological characteristic by which lymphogranuloma and psittacosis viruses can be distinguished in the laboratory.

SUMMARY.

A new method for the cultivation of psittacosis virus in the developing egg has been described, namely by inoculation into the allantoic cavity.

This route may be used for the detection of minimal amounts of virus, the method being at least as sensitive as mouse intranasal inoculation.

Large quantities of virus may be obtained by the method.

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THE INFLUENCE OF THE THYROTROPHIC HORMONE ON THE BLOOD CHOLESTEROL OF INTACT AND THYROIDECTOMIZED RABBITS FED CHOLESTEROL

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The development of a state of hypercholesterolaemia in the rabbit by oral administration of the sterol has been well established. In this animal, one consequence is the production of aortic atheroma (Bailey, 1916; Leary, 1943; Menne, Beeman and Labby, 1937; Anitschkow, 1938; Hoffmeyer and Bjornsson, 1940; Wilens, 1942). The blood cholesterol of the rat and, to a greater extent, that of the guinea-pig can also be increased by similar treatment, although in neither of these animals could lipoid infiltration of the aorta or large vessels be demonstrated. (Cook and McCullagh, 1939; cf. however, Anitschow, 1922).

Comparatively few experimental investigations have been carried out to examine the relationship between the endocrine secretions and artificially induced hypercholesterolaemia. According to Renard, Columbies and Bernardbieg (1924), thyroidectomy in the *normal* rabbit produces a rise in the blood cholesterol, whilst Fleischmann, Schumacker and Wilkins (1942), reported a marked rise in the serum cholesterol under similar conditions. Fleischmann and Schumacker (1942), considered that the blood cholesterol level is not a reflection of the cholesterol content of the body, since the marked rise following thyroidectomy in the rabbit was not accompanied by a change in the total body cholesterol of this animal. Turner, Present and Bidwell (1938), on the other hand, found only a slight hypercholesterolaemia subsequent to thyroidectomy in the normal rabbit, whilst with young rabbits Turner and Khayat (1933) observed no significant change after this operation. The latter workers also concluded that the feeding of cholesterol produces a raised blood cholesterol and atherosclerotic lesions in the rabbit regardless of the presence or absence of the thyroid gland. Shapiro (1927) observed an increase in the rate of development of atheromatous deposits following thyroidectomy in the rabbit fed cholesterol. The state of hypercholesterolaemia was found to be appreciably diminished by thyroxine or insulin, both in the intact and thyroidectomized animal (Turner, Present and Bidwell, 1938). Where prolonged cholesterol feeding to the intact animal failed to cause a rise in the blood cholesterol, ablation of the thyroid gland in this "resistant" group was found to bring about a prompt and marked increase (cf. also Turner, *et. al.* 1938). Fenz and Zell (1936), noted the effect of the thyrotrophic hormone as producing a fall in the blood cholesterol of normal rabbits, whilst Bruger and Fitz (1938), claimed, as the result of experiments with a small group of animals that the administration of this hormone over a long period, concurrently with cholesterol feeding, was responsible for an increase in the degree of atherosclerosis.

The experiments to be described in the present communication were designed to ascertain the effect of administration of the thyrotrophic hormone on artificially

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induced hypercholesterolaemia in the intact and thyroidectomized rabbit. Since the completion of this investigation Turner and DeLamater (1942) have reported a fall in the blood cholesterol of this animal under the influence of the pituitary hormone both in the presence and absence of the thyroid gland.

TABLE 1.

Effect of thyrotrophic hormone on blood cholesterol of intact hypercholesterolaemic rabbits.

Rabbit No. and sex.	Period of cholesterol feeding (days).	Blood cholesterol (max. value, overall period). mg./100 c.c.	Experiment No.	Units of hormone injected.	Blood cholesterol (mg./100 c.c.). Experimental period. 1st–7th day (mean of three consecutive lowest values).	Experimental period. 13th–22nd day (mean of three consecutive highest values)	Pre-expl. period. (mean of three consecutive values).	Fall, p.c.	Subsequent rise (p.c. pre-expl. value).	Fate of animal (Time from onset of final expt.).
290** (F)	283	3,094	A	4 × 100 + 1 × 500	82	59	246	27·1	200	Killed (83rd day)
			B	4 × 100 + 1 × 500	187	94	655	49·9	250	
			C	21 × 100 (over 23 days)	411	353	1,336*	14·2	225	
280 (M)	132	1,212	A	4 × 100 + 1 × 500	728	776‡	1,125 (9th–11th day)	—	54·6	Died (51st day)
281 (M)	86	1,404	A	4 × 100 + 1 × 500 4 × 100 + 1 × 300 (over 11 days)	1,357	1,021	—	24·7	—	Died (12th day)
325** (M)	221	1,739	A	4 × 100 + 1 × 500	73	60	199	17·8	176	Killed (56th day)
			B	4 × 100 + 1 × 500	230	343	911	—	296	
260 (M)	242	1,446	A	21 × 100 + 4 × 500 (over 29 days)	291	217	574	25·5	97·5	Killed (66th day)
309** (F)	279	1,976	A	4 × 100 + 1 × 500	96	100‡	309	—	222	Killed (184th day)
			B	4 × 100 + 1 × 500	257	213	742	17·2	188	
298 (M)	224	1,451	A	4 × 100 + 1 × 500	662	627	854	(5·3)	29·1	Died (30th day)
			B	4 × 100 + 1 × 500	401	255	601	36·4	49·9	
282 (M)	332	1,465	A	4 × 100 + 1 × 500	160	112	232	30·0	45	Killed (138th day)
			B	4 × 100 + 1 × 500	175	85	299	51·3	70·8	
284 (M)	256	1,264	A	4 × 100 + 1 × 500	423	310†	531	26·7	25·5	Died (178th day)
5 (M)	303	961	A	4 × 100 + 1 × 500	236	362‡	445 (10th–12th day)	—	88·9	Killed (84th day)
			B	4 × 100 + 1 × 500	299	289	797	(3·4)	166	

* Rose to 2,480 mg./100 c.c. on 30th day.
† Mean of *two* consecutive lowest values.
‡ Values not complete during this period.
** "Resistant" animal.

MATERIALS AND METHODS.

Rabbits of both sexes were housed in separate cages in a room artificially heated during the winter months. The body weights and blood counts of the animals were regularly tabulated. The cholesterol (0·5 gm., in 10 ml. olive oil, per animal) was mixed with the daily ration of dry bran on alternate days for overall periods extending up to eleven months. After the dry food had

been consumed the animals were given a supplement of lucerne or fresh grass. Blood samples were taken from the medial vein of the ear prior to feeding, oxalated, and 1 ml. dried on a fat-free filter paper at 37° C. The cholesterol estimations were made according to the method of Liebermann-Burchard. Weekly values were recorded for each animal previous to an experiment involving a course of injections of the thyrotrophic hormone or thyroidectomy, such treatment only being attempted subsequent to the hypercholesterolaemia having reached a steady state over a three weeks' period. During the progress of an experiment, however, estimations were made daily. Unless otherwise stated, injections of the pituitary hormone[4], by the intraperitoneal route, were given over a five day period, 3 mg., equivalent to 100 Junkmann-Schoeller guinea-pig units (Junkmann and Schoeller, 1932), being the dose for the first four days and 500 units that on the fifth day. Some of the animals received up to four courses of injections at intervals after a steady state of the blood cholesterol had again been reached. (See Tables 1 and 3).

Thyroidectomy was performed under "ether" anaesthesia, the success or otherwise of the operation being carefully checked *post-mortem*. Two of the animals (No. 282 and No. 284) after serving as members of the intact groups were subsequently thyroidectomized and resubmitted to injections of the pituitary hormone.

All animals were ultimately sacrificed by bleeding from the carotid artery. The organs were weighed and together with the aortas submitted to macroscopic and microscopic examination. Liver portions were desiccated over anhydrous calcium chloride at 37° C., and a weighed amount then ground with purified sand and extracted with chloroform for the determination of cholesterol.

RESULTS.

(a) *General Observations.*

The blood cholesterol values of the normal rabbit on our stock diet without added cholesterol were found to lie within the range of 55–80 mg. per 100 ml. and to be not significantly affected by thyroidectomy. Although the majority of the rabbits showed a marked rise in their blood cholesterol within two or three weeks after the commencement of the special diet, a number proved "resistant" as noted by Turner and colleagues (1938). This "resistant" group was included amongst the animals on which thyroidectomy was later performed (Tables 2 and 3), with the exception of Nos. 290, 309, and 325; in these cases the intact animals were injected with thyrotrophic hormone to ascertain the effect of this substance on the "resistance" (Table 1). All animals receiving cholesterol developed anaemia (as shown by blood counts) after a period of from three to six months. The red blood cells showed anisocytosis, poikilocytosis and polychromatia. The anaemia appeared at least several weeks after the level of blood cholesterol had begun to rise, and in some cases appeared to increase or remit, according as the level of the blood cholesterol rose or fell (Nos. 282, 290). A constant relationship between the degree of anaemia and the appearance of the bone marrow *post-mortem* or the degree of hypercholesterolaemia could not be established.

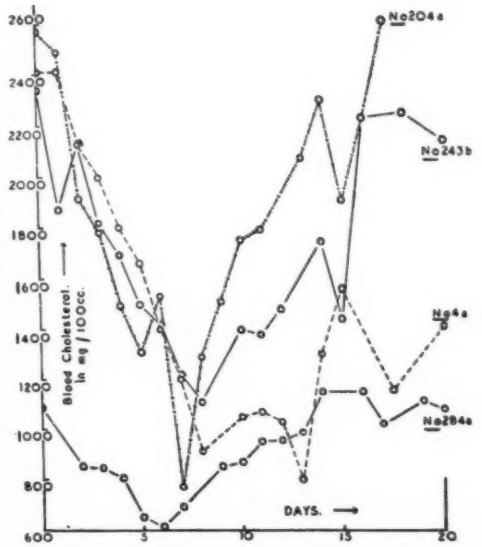

Fig. 1. The effect of thyrotrophic hormone on the blood cholesterol of thyroidectomized hypercholesterolaemic rabbits.

No general conclusions could be drawn from the histological examinations of the organs and aortas (all of which showed slight to gross atheromatous deposits) or the liver cholesterol values, with regard to their relationship either to the effect of thyroidectomy, to the administration of thyrotrophic hormone or to the degree of hypercholesterolaemia reached during the overall period of cholesterol administration.

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[4] We are indebted to Professor Schoeller, Schering A.G. for a gift of the thyrotropic hormone used in these experiments.

(b) *The Effect of Thyrotrophic Hormone on Intact Hypercholesterolaemic Rabbits.*

Ten hypercholesterolaemic intact rabbits received a course or courses of injections of the thyrotrophic factor. Altogether seventeen experiments were performed involving the administration of 900 units (4 × 100 plus 1 × 500) of the hormone, except in three instances (Nos. 290, 260, 281) where the periods of injections were much longer, and greater total doses were given. (See Table 1).

A significant fall in the blood cholesterol was observed in only eleven of the experiments, affecting eight of the animals. In the remaining six the result was either indefinite or an actual rise was indicated (No. 325, Experiment B; No. 5, Experiment B). A subsequent tendency on the part of the blood cholesterol to rise after administration of the thyrotrophic hormone was noted by Turner and DeLamater (1942) but their observations were not sufficiently prolonged. Without exception the blood cholesterol in our experiments in this group of rabbits reached within three weeks a value which was appreciably in excess of the mean value before the injections were commenced. In some rabbits the increase continued for a further period. Even in the cases of those animals receiving a prolonged course of injections the values had risen before the total dose of hormone had been administered. Subsequent to the attaining of the higher levels the values began to fall, but they continued to remain above those of the pre-experimental periods.

(c) *The Effect of Thyroidectomy.*

The very marked effect of removal of the thyroid gland on the blood cholesterol values of "resistant" and "non-resistant" hypercholesterolaemic rabbits may be seen from Table 2. One animal (No. 282) had shown a spontaneous remission of the condition of hypercholesterolaemia to a "resistant" phase (values below 100 mg. per 100 c.c.) prior to thyroidectomy (see footnote to Table 2). In this case removal of the thyroid gland resulted in a steep rise in the blood cholesterol. After a period which varied with the animal the blood cholesterol in this group became stable at the high level, which, in the case of rabbits Nos. 204 and 4 exceeded 2,000 mg. per 100 c.c. The results are in general agreement with those obtained by Turner, *et al.* (1938).

TABLE 2.

Effect of thyroidectomy on cholesterol-fed rabbits.

Rabbit No. and sex.	Period of cholesterol feeding (days).	Thyroidectomy after (days).	Blood cholesterol (mg./100 c.c.). Pre-operative. Mean of three consecutive values.	Blood cholesterol (mg./100 c.c.). Post-operative. Mean of three consecutive highest values.	Remarks.
4 (M)	250	115	73	2,368	"Resistance" abolished.
204 (M)	250	116	74	2,573	"Resistance" abolished.
230 (M)	288	71	82	1,137	"Resistance" abolished.
243 (M)	226	100	219	1,210	"Non-resistant".
296 (F)	224	115	309	1,463	"Non-resistant".
282 (M)*	332	226	99	584	"Non-resistant".
284 (M)†	256	168	551	1,168	"Non-resistant".

* No. 282 behaved in an unusual manner in that it exhibited a marked hypercholesterolaemia soon after the start of cholesterol feeding, but a spontaneous remission to a "resistant" phase then developed; the values were again elevated by injections of the pituitary principle (see Table 1) and then fell again, prior to thyroidectomy, to a low level.

† No. 284 received a course of injections of thyrotrophic hormone prior to thyroidectomy (Table 1), the operation being performed after the cholesterol level had again become stable.

(d) *The Effect of Thyrotrophic Hormone in the Thyroidectomized Hypercholesterolaemic Rabbit.*

In this group three animals, each receiving two courses of injections of the thyrotrophic hormone, showed no residual thyroid at autopsy. 900 units of the hormone were given over five or nine days as set out in Table 3. The blood cholesterol of all these animals showed in each experiment an immediate and striking fall in value, a result predictable from the marked changes in the macroscopic appearance of the blood plasma. Although the absolute quantity of cholesterol withdrawn from circulation was much greater in this group of animals than in the intact group, the percentage fall was comparable. However, a reversion to the original or higher levels did not occur as rapidly during a corresponding period in the case of the completely thyroidectomized animals, although the *total weight* of reabsorbed cholesterol was appreciable. The results of four experiments are represented graphically in Fig. 1.

TABLE 3.

(See also Fig. 1.)

Effect of thyrotrophic hormone on blood cholesterol of thyroidectomized hypercholesterolaemic rabbits.

Rabbit No. and sex.	Period of cholesterol feeding (days).	Blood cholesterol (max. value, overall period). mg./100 c.c.	Experiment No.	Units of hormone injected.	Blood cholesterol (mg./100 c.c.). Experimental period. 1st–10th day (mean of three consecutive lowest values).	Experimental period. 14th–22nd day (mean of three consecutive highest values).	Pre-expl. period. (mean of three consecutive values).	Fall, p.c.	Fate of animal (time from onset of final expt.).	Remarks.
4 (M)	250	2,484	A	9 × 100	2,413	1,057 (8th–11th day)	1,339	56·2	Killed (32nd day)	No residual thyroid
			B	4 × 100 + 1 × 500	1,557	1,042	1,347	33·2		
204 (M)	250	4,284	A	9 × 100	2,573	1,198	2,799	53·6	Killed (32nd day)	No residual thyroid
			B	4 × 100 + 1 × 500	3,682	2,714	2,840	26·3		
243 (M)	226	2,539	A	4 × 100 + 1 × 500	1,205	778	1,531	35·6	Killed (34th day)	No residual thyroid
			B	9 × 100	2,340	1,213	1,978	48·2		
284 (M)	256	1,264	A	4 × 100 + 1 × 500	1,089	657	1,099	39·6	Died (57th day)	Two small pieces of resting thyroid
296 (F)	224	1,562	A	4 × 100 + 1 × 500	851	—	—	—	Died (on 4th day)	One large piece active thyroid
282 (M)	332	1,465	A	4 × 100 + 1 × 500	564	423	351	25·0	Killed (56th day)	Two pieces active thyroid
			B	9 × 100	359	491	635	—		
230 (M)	288	1,446	A	4 × 100+ 1 × 500	1,129	1,088 (8th–11th day)	1,263	(3·63)	Killed (87th day)	One large piece of active thyroid
			B	4 × 500	1,248	1,245	1,410 (6th–9th day)	—		

Of the four rabbits remaining, on which thyroidectomy had been performed, the residual thyroid tissue was found to be in an active state in three (one of these had, however, died early in the experiment), whilst that of the fourth was of normal appearance. This last animal behaved similarly to those where thyroidectomy had been complete, whilst in only one of four experiments carried out on the other two surviving members of this sub-group was this found to be the case. (No. 282, Experiment A).

DISCUSSION.

From the results of the above experiments it may be concluded that the thyrotrophic hormone produces a fall in the total blood cholesterol of hypercholesterolaemic rabbits both in the presence and absence of the thyroid gland. In this respect the influence of the pituitary principle is similar to that of insulin (Turner, Present and Bidwell, *loc. cit.*). However, it is generally held that the changes in metabolism wrought by the pituitary hormone are mediated via the thyroid gland, the latter being stimulated to overproduction of its active principle. Thyroxine is known to reduce the blood cholesterol level in the hypercholesterolaemic rabbit and in spontaneous and post-operative clinical myxoedema, although in the latter con-

dition the thyrotrophic hormone has been shown to have no significant effect (Harrison, 1939; Robinson, 1941; see also Schnitker, Raalte and Cutler, 1936). From this point of view it must be assumed that a different set of conditions operate in the thyroidectomized rabbit fed cholesterol, and that it is not a suitable subject for comparison with the hypothyroid or thyroidectomized human being. Although the pituitary preparation used in these experiments was of high potency and was found, on assay, to be free of growth, gonadotrophic and lactogenic hormones, it was not tested for the presence of other factors.

The very marked subsequent rise in blood cholesterol observed uniformly in the intact group may signify the exercising of a compensatory mechanism, although the intervention of substances of the "anti-hormone" type would be unlikely during the short-term experiments. Of interest was the abolition of the "resistant" phase by the thyrotrophic hormone in the three animals in which this condition had been observed. No explanation of these results can, at present, be offered.

SUMMARY.

Short term injections of the thyrotrophic hormone have been shown to occasion a sharp fall in the blood cholesterol of hypercholesterolaemic rabbits, both in the presence and absence of the thyroid gland.

In the intact hypercholesterolaemic animal this initial fall was followed by a marked and prolonged elevation of the blood cholesterol level.

Thyroidectomy or injections of the thyrotrophic hormone were found to abolish the "resistance," noted in certain of the animals fed cholesterol, to the development of a state of hypercholesterolaemia.

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THE δ HAEMOLYSIN OF *CLOSTRIDIUM WELCHII* TYPE C

1. A CHARACTERISTIC HAEMOLYTIC REACTION OF *CL. WELCHII* TYPE C ON RUMINANT BLOOD AGAR.

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In 1930 McEwen described as the cause of an enterotoxaemic disease of sheep in England an anaerobic sporulating toxinogenic bacillus, greatly resembling *Bacillus welchii*, to which he gave the name *B. paludis*. McEwen found that *B. paludis* antiserum protected animals against *B. welchii* as well as against *B. paludis*, whereas *B. welchii* antiserum did not protect against *B. paludis*. Since the classificatory studies of Wilsdon (1931), *B. welchii* and *B. paludis* are now known as type A and type C, respectively, of the species *Clostridium welchii*. Wilsdon, who carefully compared the four toxinogenic types of *Cl. welchii*, reached conclusions similar to McEwen's: he found that type C did not differ in any regular and characteristic manner, either culturally or biochemically, from types A, B, or D, and could be distinguished with certainty from them only by toxin-antitoxin neutralization tests upon animals.

In this communication we describe a characteristic haemolytic reaction, present evidence that it owes its specific characteristics to the action of the δ haemolysin of Glenny *et al.* (1933), and demonstrate its value as a presumptive test for recognizing colonies of *Cl. welchii* type C. The observations were made upon 4 strains from the National Collection of Type Cultures, London (strains "3178", "3180", "3182" and "3227") and 6 from the Wellcome Physiological Research Laboratories, England ("McEwen", "Ashby", "Bates", "W49", "878" and "993"). They were compared with 2 strains of type B from the N.C.T.C. (strains "3110" and "4964"), and with a large number of strains of types A and D, many of which were freshly isolated in Australia[1]. Among the type A strains were well-known highly toxinogenic and haemolytic strains.

HAEMOLYTIC REACTION OF *CL. WELCHII* TYPE C ON BLOOD AGAR.

Type C can produce in suitable media and under appropriate conditions three distinct haemolysins. Glenny *et al.* (1933) showed that Wilsdon's four toxinogenic types A, B, C and D all produced a haemolysin referred to as α. In culture filtrates of type A they recognized the presence of another haemolysin, subsequently referred to by Macfarlane, Oakley and Anderson (1941) as θ. We found that all four types may produce θ as well as α; indeed, some strains of type C yield very large amounts of θ (Turner, Eales and Rodwell, 1942). Glenny *et al.* (1933) found that type C produced, in addition to α, a haemolysin referred to as δ, which was probably produced in very small amounts by type B also, since anti-B sera contained anti-δ.

Since type C produces in addition to α and θ haemolysins a large amount of δ, it might be expected that its haemolytic reactions would differ from those of types A, B and D, but no suggestion of this occurs in the literature; indeed, McEwen (1930) stated that "the *beta* type of haemolysis produced by *B. paludis*" was "in close agreement with the reactions of *B. welchii* described by Brown", and Borthwick (1935) found that the colonies and haemolytic reactions of types A, B, C and

[1] *Cl. welchii* types B and C have never yet been recognized in Australia.

D on Zeissler's glucose blood agar plates were indistinguishable. The species of blood used by these investigators is not stated.

We have found that if ox, goat, sheep or, less satisfactorily, pig blood is used in agar plates, the haemolytic reactions around colonies of type C are quite distinctive from those around colonies of types A, B or D.

TECHNIQUE.

The most satisfactory basal medium, in our experience, is veal infusion Witte peptone agar (= V.I.W. agar), but satisfactory results may be obtained on other basal media.

The basal medium consists of veal infusion containing 1 p.c. Witte peptone, 0·5 p.c. commercial sodium chloride and 2·25 p.c. agar; it is adjusted to pH 7·9 before autoclaving, after which the pH falls to between 7·5 and 7·7. The inclusion of 1 p.c. of phosphate buffer salts, or of 10–20 p.c. normal serum, enhances the distinctiveness of the haemolytic reaction. Although "layered plates" are not essential, the reaction is more striking on them; they also have the advantage of ensuring a uniform layer of blood agar. In order to prepare them, a layer about 2 mm. thick of V.I.W. agar or of simple 2·5 p.c. agar solution in distilled water is first poured in the plates and allowed to set; it is then covered with another 2 mm. layer of V.I.W. agar containing 10 p.c. of fresh defibrinated ox blood. The plates may be stored at 4° C. and, although best when fresh, are useful for about a fortnight.

After being inoculated on the surface, the plates are incubated anaerobically in McIntosh and Fildes' jars for 24 hours.

THE CHARACTERISTIC REACTION.

What distinguishes the haemolytic reactions of *Cl. welchii* type C from those of types A, B and D, is the presence of a wide zone of partial or almost complete haemolysis around colonies of type C, outside the narrow zone of clear haemolysis which occurs round colonies of all four types. The degree and extent of the reaction varies somewhat according to the species of blood. If the defibrinated blood is not fresh when added to the agar, the haemolytic reaction may not be very apparent until the cultures have been left on the bench aerobically for about 18 hours. On V.I.W. agar prepared with fresh defibrinated blood the following reactions are found around colonies, sown by "spot inoculation", when the plates are examined after 24 hours incubation anaerobically at 37° C.

Ox blood V.I.W. agar. With the exception of poorly haemolytic strains of types A, B and D, colonies are surrounded by a narrow zone, 1 to 2 mm. wide, of clear haemolysis. Around colonies of types A and D this zone is surrounded by another zone 2–3 mm. wide which is usually darker, but on some batches of media may be faintly lighter, than the surrounding medium; in either case the outer zone is bounded by a dark line or narrow dark band about 1 mm. wide. With the two strains of type B this outer zone is poorly developed.

On the other hand, the colonies of type C are surrounded by a similar narrow zone of clear haemolysis, which is in turn surrounded by another zone, 3–4 mm. wide, of bright pink haemolysis, which is usually not quite complete, and which is also bounded by a dark line or narrow dark band. The difference between the haemolytic reactions of type C and of types A, B and D, is thus very striking and quite unmistakable (Fig. 1).

If the plates are left aerobically at room temperature for some days, the haemolytic reactions around colonies of types A, B and D usually do not change greatly. The zones of clear haemolysis become colourless, but this is undoubtedly due to diffusion of the liberated haemoglobin throughout the medium, for colonies grown on lyzed-blood agar do not cause decolourization of the haemoglobin. On the other hand, with type C the zone of clear haemolysis gradually extends into the partly lyzed zone, which may become completely lyzed. A widening zone of partial lysis extends several mm. beyond the original dark boundary line, which may also become lyzed. The spreading zone of partial lysis is bounded by a dark red zone several mm. wide.

EXPLANATION OF FIGURE 1.

Fig. 1. Illustrating the haemolytic reactions of *Cl. welchii* types A and C on 10 p.c. ox blood veal infusion Witte peptone agar. The 3 colonies in the upper segment are type A, the 5 lower and central colonies are type C.

(a) Control plate; (b) plate containing 10 units anti-α and 0·03 units anti-θ per ml.; (c) plate containing 0·02 units anti-α and 13 units anti-θ per ml.; (d) plate containing 10·02 units anti-α and 13·03 units anti-θ per ml.; (e) plate containing 3·6 units anti-α, 50 units anti-θ and 105 units anti-δ per ml. On (d) haemolysis totally suppressed around colonies of type A and on (e) it is totally suppressed around colonies of both type A and type C (positions of the colonies are indicated by arrows). (f) Four colonies of type C (indicated by arrows), recognizable by the extra zone of partial haemolysis, on an ox blood V.I.W. plate sown with a mixture of 1 part of type-C culture to 19 parts of type-A culture. The resistance of red cells in the darkened zones around type-A colonies to haemolysis by type C is illustrated.

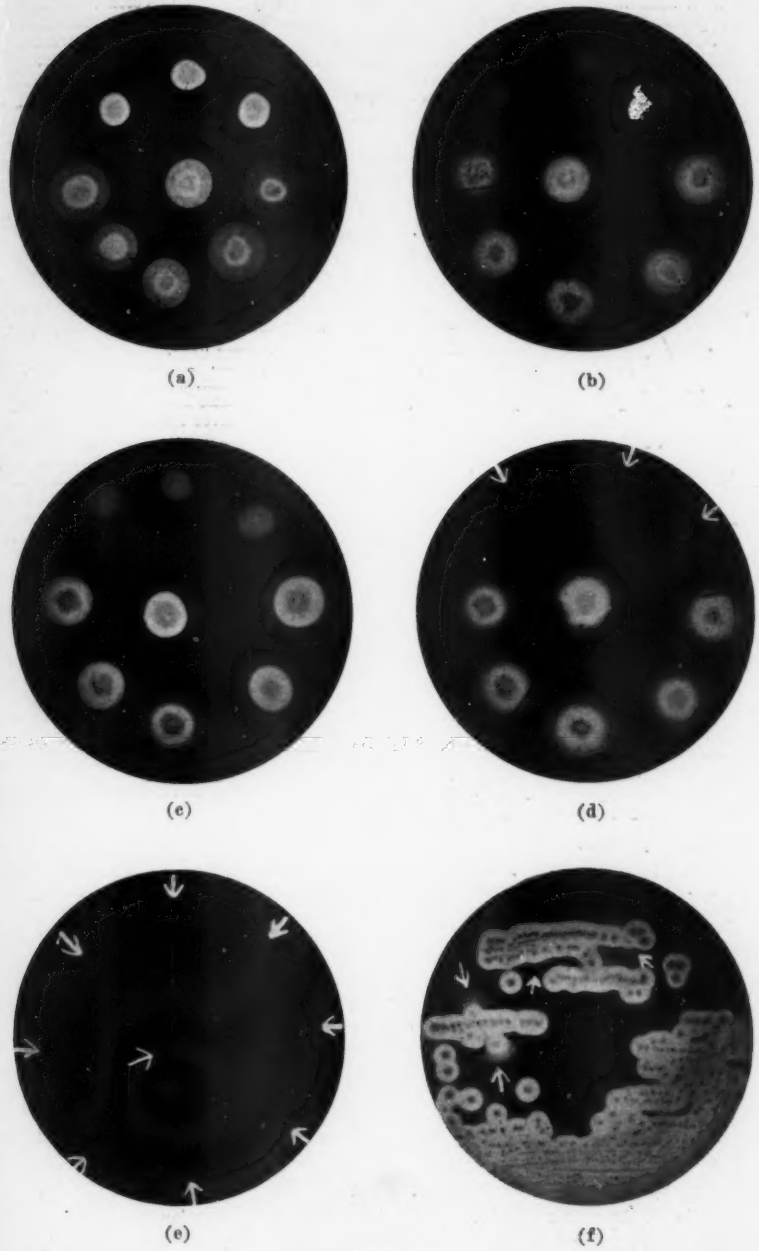

Fig 1.

The dark zone which surrounds the zone of clear haemolysis around colonies of types A, B and D is probably due to more than the mere concentration of haemoglobin liberated from the lyzed cells. Whatever its nature, it alters the cells so that they become resistant to the action of the haemolysins which diffuse from colonies of type C. This is illustrated in Fig. 1, f.

Goat blood V.I.W. agar. The characteristic outer zone of partial haemolysis around colonies of type C is a little wider than on ox blood agar, and is usually less complete when the jar is opened. After a day or so aerobically at room temperature it usually becomes completely lyzed.

Sheep blood V.I.W. agar. The characteristic outer zone is still less completely lyzed, though very easily recognized, and is even wider than on ox blood agar. The total area of lysis around "spot-inoculated" colonies of type C is about 20 mm. in diameter and is bounded by a dark line. Lysis never becomes really complete even when the plates are left aerobically at room temperature. The zone of clear haemolysis around colonies of types A, B and D is surrounded by a wide zone in which the blood is usually much darkened, but with some batches of media it may be unaltered or even slightly lightened, and is bounded by a dark line. The total area of influence may reach 14 to 16 mm. in diameter but the reaction is very readily distinguished from that around colonies of type C by the absence of haemolysis in this second zone.

Pig blood V.I.W. agar. The reactions, though not so striking as on ruminant blood V.I.W. agar, are nevertheless fairly distinctive and can be differentiated from those produced by types A, B and D. Around the colonies of all types is a narrow zone (1 mm. wide) of partial haemolysis, which is almost complete with type C but may be absent with type B. Beyond this primary zone colonies of types A, B and D develop a faint light zone from 2 to 3 mm. wide, bordered by a faint dark line. When the plates are kept aerobically for some days this zone becomes somewhat lighter With the exception of strain "3178", which on this medium behaves almost indistinguishably from strains of types A, B and D, strains of type C are distinguished by a zone of faint partial lysis which extends 2 mm. from the zone of almost complete lysis and is itself surrounded by a faintly light zone 2 mm. wide. That is to say, the zone is a little wider around type C than around colonies of types A, B and D, and with most strains it is invaded by a distinct zone of faint partial lysis. This becomes much more apparent after a day or so aerobically at room temperature, when this zone of partial lysis becomes much more lyzed. However, lysis does not proceed very far.

FACTORS INFLUENCING THE REACTION.

(a) *The Composition of the Basal Medium.*

In our experience, the optimal basal medium is undoubtedly veal infusion containing 1 p.c. Witte peptone. Combined with the optimal blood, i.e. ox blood, this has given consistently good results with all strains. With other peptones, veal infusion gives good results with some strains but poor results with others, such as strain "3178", even when combined with ox blood. Horse infusion, Lemco extract, V.F. or 4 p.c. Evans' peptone are less satisfactory than veal infusion. Fastidious strains such as "3178" presumably develop the characteristic haemolytic reaction only when essential growth substances are present in sufficient concentration: for such strains, presumably, these requirements are met by veal infusion *plus* Witte peptone *plus* the blood of ox, goat or sheep, but are not met by V.F. digest, by Lemco extract *plus* Bacto-peptone, or by horse infusion *plus* Bacto-peptone unless ox blood is used.

(b) *The Species of Blood.*

This is of prime importance. If the blood of horse, rabbit, man, or guinea-pig is used, even with V.I.W. agar, the haemolytic reaction around colonies of type C cannot be distinguished from the reactions around colonies of types A, B and D, or it differs extremely slightly.

Horse blood. The haemolytic reaction around all four types conforms in general to the description given by Topley and Wilson (1936) for the appearance of *Cl. welchii* on horse blood infusion agar: colonies are surrounded by a narrow zone of clear haemolysis. In addition, this zone in turn is surrounded by an ill-defined narrow dark zone about 1·0–1·5 mm. wide.

Rabbit blood. The four types behave similarly: there is a narrow zone of haemolysis surrounded by a very faintly light zone, 5–6 mm. wide.

Human blood. The four types are surrounded by a narrow zone of haemolysis which is bounded by an ill-defined dark zone 2–3 mm. wide. Around colonies of type C, after careful inspection, a very faint darkening may be recognized for a further 3–5 mm.

Guinea-pig blood. The four types are surrounded by a narrow zone of haemolysis. In addition, colonies of type C are surrounded by a very faintly light zone 3–5 mm. wide.

The slightly different reactions of type C from types A, B and D with human or guinea-pig blood are very difficult to detect and have little practical value.

(c) *The Necessity of Serum for the Reaction.*

At least in all the agar media investigated by us, the serum fraction of blood is essential for the reaction, since it occurs only when the medium contains both serum and red cells: whereas on agar containing 10 p.c. ox blood the haemolytic reaction around type C is characteristic and distinctive, on agar containing 5 p.c. washed ox red cells no difference can be detected between

the reactions around types A, B, C and D; in each case there is a narrow zone of clear haemolysis surrounded by a wide very faintly light zone. Increasing the concentration of serum by adding 20 p.c. of normal ox serum to the standard 10 p.c. ox blood V.I.W. agar plates enhances the distinctiveness of the reaction around colonies of type C; the zone of clear haemolysis around types A, B and D may be considerably suppressed, whereas around type C the characteristic outer zone of haemolysis, although a little reduced in width, is complete instead of partial.

(d) *The Specific Importance of Red Cells and the Non-Specificity of Serum.*

That the red cells and not the serum constituents of susceptible bloods are the important factor is shown by comparing the haemolytic reactions on V.I.W. agar plates in which are incorporated artificial "hybrid" bloods, made by adding washed ox red cells in normal proportions to horse serum and washed horse red cells to ox serum. The characteristic reaction is obtained on the ox cells *plus* horse serum plates but not on the horse cells *plus* ox serum plates.

(e) *The Effect of Cl. welchii Antisera.*

In order to determine the parts played by the 3 haemolysins α, θ and δ in the specific haemolytic reaction, antisera of a sufficient degree of purity are necessary. This was possible for anti-α and anti-θ, but not for anti-δ. We are greatly indebted to Dr. Parrish, Wellcome Physiological Research Laboratories, England, who kindly supplied the following antisera and their particulars.

Antiserum.	Units[2] of anti-haemolysins per ml.		
	α	θ	δ
LX241	100	0·3	—
R5433	0·2	130	—
R8132	36	500	1,050

These sera were added to ox blood V.I.W. agar to the extent of 10 p.c.

It was found that antiserum LX241 (≡, in each ml. of blood agar, 10 units anti-α and 0·03 units anti-θ) and antiserum R5433 (≡ 0·02 units anti-α and 13 units anti-θ) each reduced the haemolytic reactions around colonies of type A considerably, but not entirely, the inhibitory effect of LX241 being greater than that of R5433; on the other hand, they had a stimulating effect on the haemolytic reaction around colonies of type C. A combination of 10 p.c. each of LX241 and R5433 (≡ 10·02 units anti-α and 13·03 units anti-θ) completely inhibited all trace of haemolysis around colonies of type A, but again had no inhibitory effect on the haemolysis around colonies of type C; indeed, the reactions showed the usual non-specific enhancing effects due to the increased proportion of serum in the agar. On the other hand, antiserum R8132 (≡ 3·6 units anti-α, 50 units anti-θ and 105 units anti-δ) completely inhibited any haemolysis around colonies of both type A and type C. From this it appears very likely that the characteristic type-C reaction is due mostly if not wholly to δ, although it cannot be excluded that α and θ might play some part in it. Unfortunately, we had no pure anti-δ serum to make this more certain, nor a sufficiently potent anti-θ serum to enable us to prepare a mixture of anti-α and anti-θ, within the required volume, corresponding to the proportions present in antiserum R8132.

THE HAEMOLYTIC REACTION AS A PRESUMPTIVE TEST FOR *CL. WELCHII* TYPE C.

The haemolytic reactions around colonies that arise from single cells of *Cl. welchii* differ only slightly in extent from those around the "spot-inoculated" colonies described earlier, but the colonies themselves are smaller. After 24 hours' incubation the colonies are from 1·0 to 1·5 mm. in diameter and each is surrounded by a zone of clear haemolysis about 1·5 mm. wide. The outer dark zone around colonies of types A, B and D, and the bright zone of almost complete lysis around colonies of type C, may reach from 3·0 to 3·5 mm. in width. Type C is clearly distinguishable by its zone of bright, almost complete lysis, surrounding the zone of clear haemolysis. It is therefore easy to recognize colonies of type C in mixtures with other types by which it is grossly outnumbered (Fig. 1, *f*).

It is true that colonies of type C are usually somewhat flatter and less regular than those of types A, B and D but this has little determinative value. Therefore without a criterion such as this specific haemolytic reaction, the procedure when attempting to determine the presence of *Cl. welchii* type C would be to isolate as many "welchii-like" colonies, selected at random, as could be investigated, to grow each in liquid media suitable for developing the specific lethal toxins of type C and to carry out laborious toxin-antitoxin neutralization tests upon mice. The probability of including at least one colony of type C among any 6 colonies distributed randomly on a plate containing 10 colonies of type C and 190 of types A, B or D is only 0·265. With the aid of this haemolytic reaction, even a single typical colony of type C could be recognized with certainty from, say, 199 colonies of other types, whereas without such assistance the probability of includ-

[2] α is in International units, the others are in W.P.R.L. provisional units.

ing it among any 6 colonies selected at random would be only about 0·03. The saving of effort and increased certainty of isolation are thus very great.

We have successfully applied the technique to isolating type C from the faeces of rabbits to which it had been fed.

DISCUSSION.

It seems possible that this striking haemolytic reaction of *Cl. welchii* type C has not been observed and recorded by other investigators because they have not used ruminant blood in preparing their blood agar plates.

That the characteristic part of the haemolytic reaction around colonies of type C is due to a readily diffusible haemolysin which is not produced by colonies of types A, B and D is suggested by the characteristic progressive spreading lysis which occurs even after the plates are removed from the anaerobic atmosphere. *A priori* one would expect that it is due to the δ haemolysin which this type is known to produce in addition to α and θ. This is supported by the observation that a mixture of anti-α and anti-θ sera, while suppressing entirely the haemolysis around colonies of types A, B and D has only a slight effect on the haemolysis around colonies of type C, whereas if the mixture includes anti-δ the haemolysis is prevented around colonies of type C also. We have found that serum is essential for the production of the haemolytic reaction on blood agar plates, but there is no evidence in the literature that English workers have found serum essential for the production of δ in liquid media. Nevertheless, that production of δ requires special nutritive conditions is obvious from the failure of Mason, Ross and Dalling (1931) and of Mason (1935) to demonstrate it when they used common laboratory media, and in a further communication of this series we shall show that in nearly all media examined by us very little or no δ is produced unless serum is added. Like Dalling and Ross (1938) we were successful in demonstrating large yields of δ in horse infusion peptone broth to which 10 p.c. of the extracted meat had been added; but we found that in this medium its appearance is very fleeting, reaching a maximum after a few hours' growth and then disappearing, unless serum is added. In this case serum appears to prevent rapid deterioration of δ. Even on ruminant erythrocyte agar prepared from the liquid portion of this medium, we found that the characteristic type-C haemolytic reaction was not produced unless serum was present. Therefore it seems that, on media containing agar, δ is not produced or at any rate is not active unless serum is present.

It is implicit in the Wilsdon classification of the *Cl. welchii* group that the criteria for typing are the results of toxin-antitoxin neutralization tests upon animals and are therefore concerned only with the lethal toxins. There is no evidence that δ has lethal properties, at any rate in the usual test animals (mice, guinea-pigs or rabbits). The identification of Wilsdon's *Cl. welchii* type C must therefore finally rest, by definition, upon the demonstration that the lethal activity of its growth products is neutralized by both anti-B and anti-C sera but not by anti-A or anti-D sera.

The presumptive value of the haemolytic reaction described above must largely depend upon whether it is always associated with the lethal toxins characteristic of type C. The possibility must be admitted that there may exist "degraded" strains originally belonging to type C, which have lost their capacity to produce the lethal β and γ toxins but which are still able to produce δ haemolysin. Indeed Taylor (1940) described the loss of capacity to produce β toxin by certain cultures of the "CT40" strain, of which our "3178" is a sub-culture. We have observed the same phenomenon with this strain, and have isolated from it a variant which, although unable to produce the characteristic lethal toxin β, is still able to produce the haemolytic reaction. It is possible to imagine another type of degradation resulting in the loss of capacity to produce δ haemolysin by otherwise typical type-C strains, but we have never observed this.

Finally, since some strains of type B are said to produce demonstrable although small amounts of δ, strains may exist which are able to produce sufficient δ haemolysin to cause the characteristic haemolytic reaction, but we have never observed this, even when the ox blood plates were reinforced with 20 p.c. ox serum.

Oakley (1943) has rightly said "What is really important is not what strain is present, but which toxins it can produce, and, were not the usual division of *Cl. welchii* into types so practically convenient, there would be much to be said for abandoning it, and referring to filtrates as containing α, β, γ, δ, ϵ, θ, η toxins according to which were present." The haemolytic reaction described in this communication is merely an indicator of the production of δ haemolysin, but in spite of the possibilities discussed it can be taken as highly presumptive evidence of *Cl. welchii* type C and it enormously facilitates its recognition and isolation.

Surveys to determine the distribution of the various types of *Cl. welchii* have been very limited, mainly through technical reasons: the amount of labour, material and animals required is very great. By taking advantage of the specific haemolytic reaction described herein it should prove comparatively easy to carry out surveys for *Cl. welchii* type C, concerning which very little information yet exists.

SUMMARY.

When grown on the surface of suitable agar media containing 10 p.c. of ruminant (ox, goat, sheep) blood, colonies of *Cl. welchii* type C are surrounded, in addition to the narrow zone of complete haemolysis which is common to the four Wilsdon types, by an outer wide zone of almost complete haemolysis which sharply disinguishes them from the colonies of types A, B and D. Inferior but essentially similar reactions are produced with pig blood. The reaction does not occur with the blood of horse, rabbit, man or guinea pig.

It is produced only when the agar medium contains serum as well as red cells, but the sera of species whose red cells are not susceptible are effective in combination with the red cells of susceptible species.

The characteristic zone of almost complete haemolysis is inhibited by mixtures of anti-α, anti-θ and anti-δ sera but not by mixtures of anti-α and anti-θ sera which completely inhibit the haemolysis around colonies of types A, B and D. The evidence therefore points to its being caused by δ haemolysin.

The reaction is only an indicator of the presence of δ haemolysin, but in practice it may be taken as highly presumptive of *Cl. welchii* type C and it greatly facilitates its recognition and separation from types A, B and D.

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